An Investigation into the Use of Lumpy Skin Disease Virus as a Vaccine Vector for a Potential HIV-1 vaccine

By

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Declaration

The study described in this thesis was performed in the Division of Medical Virology, Department of Clinical Laboratory Sciences of the University of Cape Town, under the supervision and guidance of Professor Anna-Lise Williamson, Professor Enid G Shephard and Dr Nicola Douglass. This is my own work and the use of information from others has been referenced. The assistance received from others has also been acknowledged.

Signed by candidate

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Yen-Ju Shen

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List of abbreviations

Ad5, 11, 36	Adenovirus serotype 5, 11, 36
AIDS	Acquired immune deficiency syndrome
BAC	Bacterial artificial chromosome system
β-gal	β-galactosidase
Bp	Base pair
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CAM	Chorioallantoic membrane
CBA	Cytometric Bead Array
CD	Cluster of differentiation
CEV	Cell-associated enveloped virus
ConA	Concanavalin A
CPE	Cytopathic effects
CRFs	Circulating recombinant forms
CSPG	Chondroitin sulfate proteoglycan
CTL	Cytotoxic T lymphocytes
DMEM	Dulbecco's Modified Eagle medium
DNA	Deoxyribonucleic acid
EEV	Extracellular enveloped virus
EFC	Entry/fusion complex
ELISPOT	Enzyme-linked immunosorbent spot assay
FBT	Foetal bovine testes
FCS	Foetal calf serum
ffu	Focus forming unit
Flt-3L	Fms-like tyrosine kinase 3 ligand
g	Gravitational force or gram
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GMCSF	Granulocyte-macrophage colony stimulating factor
GMP	Guanosine monophosphate
Gpt	Guanine-hypoxanthine phosphoribosyltransferase
Grttn	Gag, reverse transcriptase, Tat and Nef polyprotein
GUS	β-glucuronidase
HA	Hemagglutinin
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycan
IEV	Intracellular enveloped virus
IFN	Interferon
IL	Interleukin
IMP	Inosine monophosphate
IMV	Intracellular mature virus
ITR	Inverted terminal repeats
IV	Immature virion

Kb	Kilobase
kDa	Kilodaltons
LAMP	Lysosomal-associated membrane proteins
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
m.o.i	Multiplicity of infection
MDBK	Madin-Darby bovine kidney epithelial cells
µg	Microgram
µl	Microlitre
mg	Milligram
ml	Millilitre
mM	Millimolar
MPA	Mycophenolic acid
MVA	Modified vaccinia Ankara
ng	Nanogram
NHP	Non-human primate
PBS	Phosphate buffered saline buffer
PCR	Polymerase chain reaction
Pent	Pentamer
pfu	Plaque forming unit
PR	Protease
RBC	Red blood cells
rLSDV	Recombinant lumpy skin disease virus
rLSDV-Grttn	Recombinant lumpy skin disease virus expressing Grttn
rMVA	Recombinant modified vaccinia Ankara
rMVA-Grttn	Recombinant modified vaccinia Ankara expressing Grttn
rpm	Revolutions per minute
RPMI	Royal Park Memorial Institute culture medium
RR	Ribonucleotide reductase
RT	Reverse trascriptase
RVFV	Rift valley fever virus
SAAVI	South African AIDS Vaccine Initiative
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	Serum-free medium
SFU	Spot forming unit
SHIV	Simian/human immunodeficiency virus
SIV	Simian immunodeficiency virus
TAE	Tris-acetate-EDTA
T cell	T-lymphocytes
TK	Tyrosine kinase
TNF	Tumour necrosis factor
TSEs	Transmissible spongiform encephalopathies
UV	Ultra violet light
VLPs	Virus-Like-Particles
VSV	Vesicular stomatitis virus

VV	Vaccinia virus
WHO	World Health Organization
wt	Wild type
wtLSDV	Wild-type lumpy skin disease virus
wtMVA	Wild-type modified vaccinia Ankara
XMP	Xanthosine monophosphate
YT	Yeast- Tryptone

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Abstract

With 33.4 million people infected with human immunodeficiency virus (HIV) in 2008, HIV infection has developed into a serious global pandemic. The impact of the epidemic is most severe in developing countries, such as the countries in Sub-Saharan Africa, where social and economic conditions in these regions affect the availability of antiretroviral treatments. The control of the epidemic in these regions could be achieved with an effective HIV vaccine. The urgent need for an efficacious HIV vaccine as well as the requirement for vaccine vectors for other infectious diseases, provide the rationale for research into novel vaccine vectors that are both safe and efficacious.

Lumpy skin disease virus (LSDV) Neethling strain, a capripoxvirus with host range limited to ruminant animals, is unable to complete its replication cycle in human cells. LSDV has been demonstrated to be a safe and effective veterinary vaccine vector for lumpy skin disease as well as a vaccine vector for other infectious diseases of animals. In this thesis LSDV was investigated to determine if it would be a suitable HIV vaccine vector. As no data was available on the safety profile of LSDV in immunocompromised hosts, the safety of wtLSDV was evaluated in two strains of immunocompromised mice in this study. This study also describes the construction of a recombinant LSDV, rLSDV-Grttn, expressing an HIV-1 subtype C polyprotein, Grttn, (comprised of Gag (G), reverse transcriptase (RT), Tat and Nef) and the testing of this recombinant for immunogenicity to Grttn in BALB/C mice.

Immunocompromised mice (RAG and CD4 knockout) survived during a 30 day observation period after vaccination with wtLSDV (10^4 ffu and 10^6 ffu/mouse) and did not experience any significant weight change or change in well being compared to naïve control mice during this period.

rLSDV-Grttn was constructed through introduction of the Grttn gene into the ribonucleotide reductase gene of LSDV. Stable Grttn expression (from the VVmH5 promoter) was confirmed by Western Blot analysis and PCR analysis showed the final rLSDV-Grttn preparation to be free from wild type LSDV.

Immune responses to this candidate vaccine were investigated using female BALB/c mice. rLSDV-Grttn was investigated as an individual vaccine and in prime/boost combinations with a DNA vaccine, pVRCgrttnC, or a recombinant MVA, MVA-Grttn, vaccine. Both vaccines express the Grttn antigen, identical to that expressed by rLSDV-Grttn. Gag- and RT-specific

immune responses induced by these regimens were evaluated with IFN-γ and IL-2 ELISPOT assays and CD8+ T cell MHC-I peptide pentamer binding assays. The presence of Gag-specific antibodies in the sera of vaccinated mice was assessed using the LAVBlot I assay. (NO RT data given)

rLSDV-Grttn alone was shown to induce low cumulative Gag- and RT-specific IFN-γ (131 net SFU/10^6 splenocytes) and IL-2 (50 net SFU/10^6 splenocytes) ELISPOT responses. These responses were enhanced when rLSDV-Grttn was used as a boost after a prime with pVRCgrttnC (607 and 110 net IFN-γ and IL-2 SFU/10^6 splenocytes respectively). However the IFN-γ and IL-2 ELISPOT responses induced by pVRCgrttnC primed/ rMVA-Grttn boosted mice were 2.2-2.5 times higher (1529 and 245 net SFU/10^6 splenocytes respectively) than pVRCgrttnC primed/rLSDV-Grttn boosted mice. When rLSDV-Grttn was evaluated in heterologous prime/boost vaccination regimens with rMVA-Grttn, Gag- and RT-specific cumulative IFN-γ and IL-2 ELISPOT responses were higher than that of the pVRCgrttnC prime/rMVA-Grttn boost regimen. A rLSDV-Grttn prime and rMVA-Grrtn boost induced Gag- and RT-specific cumulative IFN-γ and IL-2 ELISPOT responses of 3067 and 449 net SFU/10^6 splenocytes respectively. The reverse regimen i.e. rMVA-Grrtn prime/ rLSDV-Grttn boost, induced similar Gag- and RT-specific cumulative IFN-γ and IL-2 ELISPOT responses of 2593 and 748 net SFU/10^6 splenocytes respectively. Binding of pentameric H-$2D^k$ complexes folded with the Gag AMQMLKDTI peptide and the RT peptide VYYDPSKDLIA to CD8+ T cells in the spleens of the mice was used to assess the total number of HIV-specific CD8+ T cells generated by the heterologous poxvirus vaccination regimens. When rLSDVGrttn was the booster vaccine the sum of Gag- and RT- specific CD8+ T cells reached a frequency of 4.6% of the CD8+ T cells while this frequency was 7% of the CD8+ T cells when rMVAGrttn was the booster vaccine. Approximately 98% of these HIV-specific CD8+ T cells for both vaccination regimens expressed CD44 confirming these cells to be antigen experienced. The degranulation marker CD107a/b was also expressed on the majority of these HIV-1 specific CD8+ T cells (>75%). Comparison of this direct enumeration of HIV-specific CD8+ T cells with the enumeration of HIV-specific CD8+ T cells producing IFN-γ and IL-2 using ELISPOT assays, suggested that these vaccine regimens also induce HIV-specific CD8+ T cells that produce cytokines other than IFN-γ and IL-2. Gag-specific antibody was also produced in all rLSDV-Grttn/rMVA-Grttn heterologous prime-boost vaccination regimens.

In summary, LSDV Neethling strain has been shown to be a safe vaccine vector, even in *in vivo* immunocompromised settings. rLSDV-Grttn that was constructed was immunogenic in BALB/c mice, and induced HIV-1 specific immune responses, especially when used in prime-boost

vaccination regimens with rMVA-Grttn. These observations indicate that rLSDV-Grttn could be a novel and safe candidate HIV-1 subtype C vaccine, to be used in combination with recombinant MVA vaccine to induce HIV-1 immune responses that could be superior to the more commonly used DNA/MVA vaccination regimens.

Chapter 1: Literature Review

1.1 HIV-1 Vaccinology

1.2 Poxviruses

1.3 Project Motivation

1.1 HIV-1 Vaccinology

1.1.1 The need for an HIV-1 vaccine

Since the initial isolation of the Human immunodeficiency virus (HIV) in 1983 (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983), HIV infection has developed into a serious global pandemic. According to the UNAIDS 2009 reports (UNAIDS, 2009), the numbers of people infected with HIV and who have died from HIV-related causes are estimated to be 60 million and 25 million people respectively since the discovery of the virus. In 2008, it was estimated that 33.4 million people were infected with HIV and there were 2 million HIV-related deaths globally. According to the estimated data from UNAIDS, there is a trend of stabilisation of the HIV pandemic, reflecting the impact of antiretroviral treatment and changes in sexual behaviour of certain population groups; however, high numbers of newly infected individuals (2.7 million) and cases of Acquired immune deficiency syndrome (AIDS) related deaths (2 million) were still observed in 2008. Although anti-retroviral therapy has a significant impact on limiting HIV transmission and controlling disease progression, it has had limited impact on developing countries where resources of antiretroviral drugs are limited (Giuliano & Vella, 2007; UNAIDS, 2009) . It is these regions which are worst affected by the HIV pandemic, in particular, Sub-Saharan Africa, which is the most affected region by the pandemic (Figure 1.1). This region accounts for 67% of the global HIV infected population and 70% of the AIDS related deaths globally. HIV-1 infections have lowered the average life expectancy in Sub-Saharan Africa to below 50 years and, up until 2008, HIV-1 related deaths have orphaned nearly 14 million children in Sub-Saharan Africa (UNAIDS, 2009) . Consequently, huge social and economic burdens have been created in this region (Mosam & Dlova, 2006). Although there has been an increase in the availability of anti-retroviral therapy within this region and a significant reduction in HIV-related deaths over the past six years (UNAIDS, 2009), the HIV pandemic remains uncontrolled in the region. Drawbacks of antiretroviral treatment including cost, diagnosis of stages of HIV-associated clinical disease, toxicity and drug resistance have limited the effect of the treatment on controlling the pandemic.

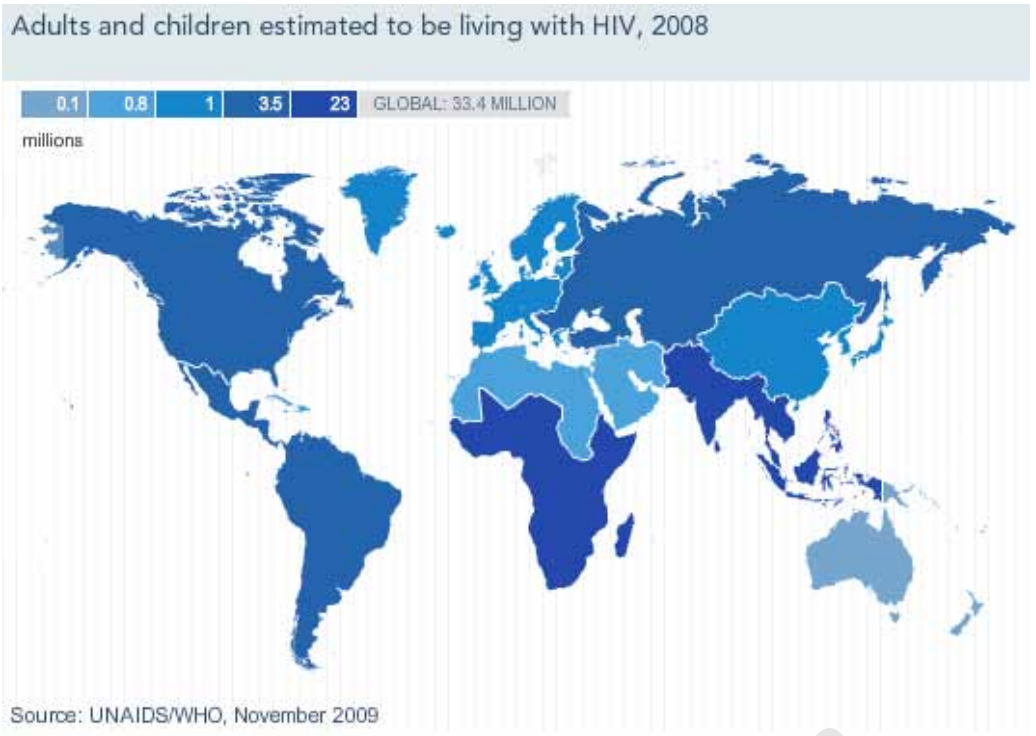

Figure 1.1 Schematic representation of global HIV-1 prevalence in 2008 (UNAIDS, 2009). The different shades of blue represents different prevalence population categories in millions people.

An effective prophylactic or therapeutic HIV vaccine that can either prevent HIV infection or delay disease progression to AIDS is urgently needed. Vaccination has been demonstrated to be the most effective preventative measure against infectious diseases, as was shown in the successful eradication of smallpox (Henderson, 1980) and the ongoing polio and measles vaccination campaigns (de Quadros *et al.*, 1998; Rey & Girard, 2008).

1.1.2 Evidence supporting the use of a vaccine to control the HIV-1 epidemic

1.1.2.1 Evidence from control of natural HIV-1 infection in humans

The potential ability to control the HIV-1 epidemic by vaccination is supported indirectly by what has been observed to occur in the natural course of infection with HIV-1, with respect to the specific immune response elicited by the host and its ability to control HIV-1 replication and disease progression.

The typical clinical progression of HIV-1 infection is illustrated in Figure 1.2. During the early stage of HIV-1 infection there is a burst of viraemia approximately 3 weeks post exposure. The viral load decreases until it reaches the viral set point 2 to 6 months post exposure. The reduction of viral load is believed to be attributed to the HIV-1 specific immune responses elicited by mainly the cytotoxic CD8+ T lymphocyte response (CTL) (Johnston & Fauci, 2007; Koup *et al.*, 1994) (Figure 1.2).

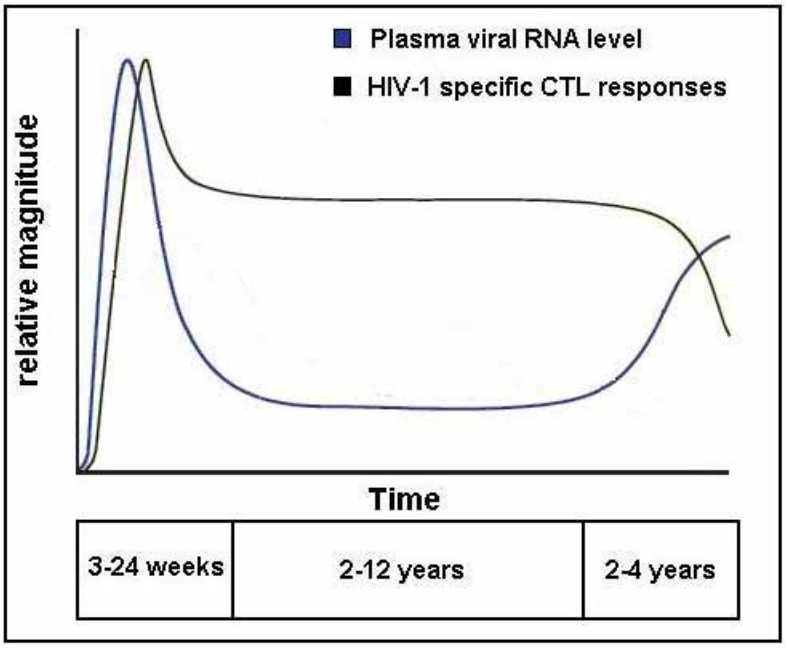

Figure 1.2 Schematic representation of typical clinical progression of HIV-1 viral RNA and HIV-1 specific CTL in HIV-1 infected individuals (Johnston & Fauci, 2007).

Long term control of HIV-1 replication and disease progression exist in small groups of individuals in the general population. These individuals, termed HIV-1-infected elite controllers, exhibit spontaneous control of HIV-1 infection (Buchbinder *et al.*, 1994; Pereyra *et al.*, 2008). Despite the absence of antiretroviral treatment, these individuals were able to control the HIV-1 plasma viral load to <50 copies/ml for at least 12 months. The immune correlation to the control of disease progression remains unclear; however, studies have indicated that most of these individuals preferentially exhibit HIV-1 Gag-specific CTL responses and enhanced polyfunctional T cell functions (by cytokine secretions), which may contribute to their ability to contain HIV-1 replication (Miura *et al.*, 2009; Pereyra *et al.*, 2008).

Human leukocyte antigen class 1 (HLA-1) molecules are known to be associated with antigen presentation to cytotoxic CD8+ T lymphocytes. Certain HLA class I molecules, such as HLA-B57, B5801 and B27 are over-represented in elite controllers (Migueles *et al.*, 2000; Pereyra *et al.*, 2008) and are associated with control of HIV-1 related disease progression. These HLA class I molecules recognize unique Gag epitopes and exert selection pressure for escape variants with compromised viral fitness (Miura *et al.*, 2009). A large percentage of the elite controllers also express other HLA class I molecules that are not associated with viraemic control (Pereyra *et al.*, 2008). CD8+ T cell responses preferentially targeting Gag epitopes have been observed in most of the elite controllers, while the CD8+ T cell responses of progressors were found to be more broadly distributed across the different HIV-1 proteins (Pereyra *et al.*, 2008). Elite controllers have more CD4+ and CD8+ T cells that produce IFN-γ and IL-2 compared to progressors

(Pereyra *et al.*, 2008). Strong CTL responses against the escape variants help to delay the disease progression (Miura *et al.*, 2009). A strong HIV-1 neutralizing antibody response was not observed in the elite controllers, demonstrating that neutralizing antibody may not be critical to HIV-1 viraemic control (Pereyra *et al.*, 2008). It must be noted that the responses observed in the individuals in the elite controller groups were heterogeneous and none of the observations can be used to accurately predict the clinical outcome of HIV-1 infection.

These findings not only provide evidence that immunological control of HIV-1 replication is possible with the use of a vaccine; they also provide direction for future HIV-1 vaccine design.

1.1.2.2 Evidence from vaccine induced protection in non-human primates

More direct evidence indicating the effectiveness of the use of vaccines in the prevention and control of HIV-1 replication has been presented in several pre-clinical studies using simian immunodeficiency virus (SIV) or chimeric SIV expressing HIV-1 Env (SHIV) in non-human primate (NHP) models (Genesca *et al.*, 2008; Jia *et al.*, 2009; Reynolds *et al.*, 2008; Whitney & Ruprecht, 2004; Yankee *et al.*, 2009).

Neutralizing antibodies have been demonstrated to be effective in preventing infection and for control of viral replication (Chaudhri *et al.*, 2006; Hessell *et al.*, 2009; Johnson *et al.*, 2009; Mack *et al.*, 1972; Panchanathan *et al.*, 2006; Panchanathan *et al.*, 2008; Trkola *et al.*, 2008). However, no HIV-1 vaccine candidate has yet successfully elicited broadly neutralising antibody. Nevertheless, the protective property of neutralising antibody has been demonstrated in NHP models; by passive introduction of SIV- or SHIV-specific neutralizing antibodies into rhesus macaques, the viral set point was reduced, CD4+ T cells were preserved and the macaques were protected against pathogenic SIV (SIVmac239) and SHIV infection respectively (Nishimura *et al.*, 2003; Ruprecht, 2009; Yamamoto *et al.*, 2007).

Complete or partial protection from lentivirus infection afforded by live attenuated SIV vaccination was also demonstrated in NHPs (Genesca *et al.*, 2008; Jia *et al.*, 2009; Reynolds *et al.*, 2008; Whitney & Ruprecht, 2004; Yankee *et al.*, 2009). Rhesus Macaques vaccinated with live attenuated SIV were completely protected from a challenge of the pathogenic wild-type SIV (the strain from which the vaccine was derived) (Daniel *et al.*, 1992). Partial protection, which resulted in lower plasma viral load, was also achieved in the attenuated SIV vaccinated macaques challenged with heterologous strains of pathogenic SIV and SHIV (Jia *et al.*, 2009; Wyand *et al.*, 1996; Wyand *et al.*, 1999). Investigation into the NHPs immune responses induced by attenuated SIV also revealed the importance of the Gag-specific CTL response in the

macaques, which correlated with that observed in human elite controllers. This may be attributed to the timing of HIV-1 specific gene expression. Presentation of Gag epitopes by infected CD4+ T cells to CD8+ T cells occurred approximately 2 hours post infection, while the presentation of Env epitopes only occurred after the de novo synthesis of the Env protein much later in the course of infection (Mansfield *et al.*, 2008; Sacha *et al.*, 2007). This implies that the Gag in the virus may be presented before de novo synthesis of the protein.

The protection against human or simian lentiviruses afforded by the host immune responses or vaccination provides proof of principle that the control or even prevention of HIV-1 infection is possible with the appropriate vaccine.

1.1.2.3 Evidence from the RV144 Phase 3 efficacy trial in Thailand

Currently there has only been one HIV vaccine efficacy trial that has shown moderate protection from HIV infection (Rerks-Ngarm *et al.*, 2009). The vaccine regimen used in the trial was comprised of 4 priming vaccinations of recombinant canarypox virus ALVAC-HIV and two boosting vaccination of recombinant gp120 protein AIDSVAX B/E. The vaccine regimen efficacy analysis was evaluated in 8197 test subjects who received vaccination and 8198 test subject who received placebo. The analysis has shown that the vaccine regimen has a moderate protective efficacy of 31% when the number of HIV-1 infections in the vaccinated group and the placebo group were compared. However the vaccine regimen did not induce a reduction in HIV-1 viremia or CD4+ T cell loss in those vaccinated subjects who later became infected with HIV-1. The correlates of protection from this trial are still being determined (Rerks-Ngarm *et al.*, 2009).

1.1.3 Challenges of HIV-1 vaccine development

Twenty-five years have passed since HIV-1 was described (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983); there has been only one clinical trial that has shown a low level of protection from HIV infection (Rerks-Ngarm *et al.*, 2009). This vaccine is not good enough to market and so there is still no efficacious HIV-1 vaccine commercially available, despite numerous studies and clinical trials carried out in the field of HIV-1 vaccine research (Corey *et al.*, 2009; Flynn *et al.*, 2005; McElrath *et al.*, 2008; Pitisuttithum *et al.*, 2006). The absence of an ideal HIV-1 vaccine may be attributed to various scientific obstacles and the natural immune evasive mechanism of HIV-1. An ideal HIV-1 vaccine would be able to prevent HIV-1 infection by stopping the systemic establishment of the virus and would protect individuals against a wide range of HIV-1 isolates. This would most probably be achieved by a vaccine eliciting both cellular and humoral immune responses. (Johnston & Fauci, 2008). Such a vaccine has not yet been developed (Flynn

et al., 2005; Pitisuttithum *et al.*, 2006; Robertson *et al.*, 2008).

1.1.3.1 HIV diversity

HIV is thought to be derived from SIV, a virus of chimpanzees, which crossed species and became adapted to humans (Gao *et al.*, 1999). There are two types of HIV, HIV-1 and HIV-2. For the purpose of this thesis, only HIV-1 will be discussed as this is the prevalent HIV type in South Africa. HIV-1 is subdivided into three different groups, Major (M), Outlier (O) and Non-major and non- outlier (N) (Korber *et al.*, 2000), based on the differences in their genomes. Most of the circulating HIV-1 belongs to the group M. Group M is further divided into different subtypes or clades - A, B, C, D, F, G, H, J and K. Genetic variability between these subtypes are not evenly distributed throughout the genome. Genetic sequence variability within a subtypes is approximately 15%–20%, and between the subtypes 25%-35%, with the highest variability found in the Env sequences (Hemelaar *et al.*, 2006; McCutchan, 2000). The diversity in HIV-1 is mainly due to the high mutation rate resulting from the lack of proof-reading activity in the viral reverse transcriptase (RT) enzyme (Mansky & Temin, 1995). Genetic recombination also occurs between subtypes, as demonstrated by phylogenetic analysis of certain HIV-1 isolates (Hemelaar *et al.*, 2006; Robertson *et al.*, 1995). Genetic analysis has revealed clustering of differences within the genome. These clusters are found in different HIV-1 subtypes suggesting that recombination occurred between different subtypes, probably in individuals infected with multiple subtypes of HIV-1. These mosaic HIV-1 isolates are termed circulating recombinant forms (CRFs) (Robertson *et al.*, 1995).

The epidemiology of HIV-1 has shown that subtypes are not evenly distributed throughout the world, but that different HIV-1 subtypes dominate specific geographical regions (Figure 1.3). The predominating subtype in a region is determined either by a founder effect or by the transmission route common in the region. The most predominant subtypes are A, B and C (McCutchan, 2000; Peeters & Sharp, 2000). Subtype A predominates in regions such as eastern and central Africa and eastern European countries. Subtype B predominates in western and central Europe, America and Australia. Subtype C predominates in Sub-Saharan Africa, Russia and China. Several CRFs are becoming increasingly prevalent in certain areas - CRF01-AE and CRF02-AG are the recombinant isolates predominantly found in South East Asia and the western part of Central Africa respectively (McCutchan *et al.*, 1999; Motomura *et al.*, 2000; Peeters & Sharp, 2000; Piyasirisilp *et al.*, 2000).

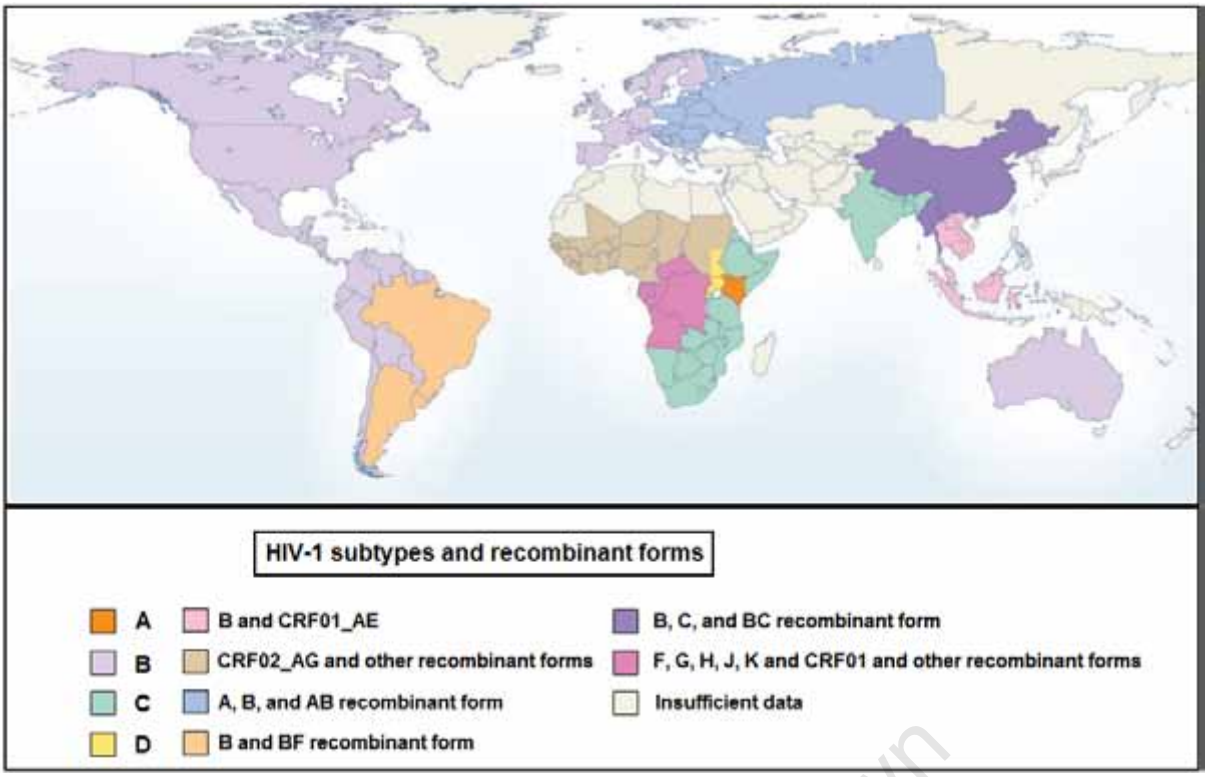

Figure 1.3 Global distributions of HIV-1 subtypes and recombinant forms (Taylor *et al.*, 2008).

The evolving diversity of HIV-1 presents a major obstacle and a moving target for developing an ideal HIV-1 vaccine that will be effective against most circulating strains. Although intra- and inter-subtype CTL cross-reactivity has been observed in HIV-1 infected patients (Brown *et al.*, 2006b; Thakar *et al.*, 2005), the magnitude and breadth of these responses are generally limited (McKinnon *et al.*, 2005). Most of the vaccine candidates designed to date have targeted single HIV-1 subtypes that dominate a specific region. However, two strategies have been deployed in the field of vaccine antigen design to try and overcome the problems of HIV-1 diversity. The one strategy entailed designing vaccine antigens from inter-subtype consensus or ancestral sequences, so that an immune response could be elicited to conserved and common epitopes within these subtypes (Bansal *et al.*, 2006; Malm *et al.*, 2005; Santra *et al.*, 2008). The other strategy entailed co-administering a mixture of vaccine vectors expressing antigens derived from different HIV-1 subtypes. Both strategies induced broad immune responses in mice and NHPs against multiple clades of HIV-1 (Brave *et al.*, 2007; Seaman *et al.*, 2005)

1.1.3.2 HIV-1 immune evasive mechanisms

The HIV-1 genome consists of nine open reading frames encoding structural proteins (Gag and Env), enzyme (Pol) and regulatory (Tat and Rev) and accessory (Vpr, Vpu,Vif and Nef) proteins (Tripathi & Agrawal, 2007). The structure of the HIV-1 virion is shown in Figure 1.4. The envelope proteins are positioned on the outside of the HIV-1 virion. The precursor env protein gp160 is cleaved into surface glycoprotein gp120 and transmembrane protein gp41 by a viral

protease. gp120 is non-covalently associated with gp41 to form heterodimers. These heterodimers are organized into trimeric "spikes" that are anchored on the viral surface membrane (Kowalski *et al.*, 1987; Weiss *et al.*, 1990). These glycoproteins are essential for binding to membrane receptors on the target cells and facilitating the fusion between viral membrane and cell plasma membrane, allowing the entry of the HIV-1 virion into the cells (Chan & Kim, 1998; Weissenhorn *et al.*, 1999; Wyatt & Sodroski, 1998). The interaction between gp120, CD4 receptor and co-receptors such as chemokine receptors CCR5 and CXCR4 on CD4+ T cells (Berson *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996), triggers a change in conformation of the trimeric complex and exposes the hydrophobic binding surface of gp41 to the host plasma membrane (Hart *et al.*, 1991; Jones *et al.*, 1998).

Because of the positioning of Env proteins on the surface of the virus and the critical role of these glycoproteins in viral entry into the target cells, they serve as primary targets for neutralizing antibody and CTL responses of the host to prevent and clear HIV-1 infection. However HIV-1 exhibits several evasive mechanisms to escape the host immune responses. The outer surface of gp120 is heavily glycosylated with N-linked glycan (Leonard *et al.*, 1990), and forms a glycan shield that blocks the binding of neutralizing antibody. The glycosylation on Env protein is an evolving mechanism. Mutation in the Env gene can shift the glycosylation sites on this protein and thus change the organization pattern of the glycan shield. The conformational change in glycan shield pattern can mask neutralizing antibody binding sites and allow the virus to escape without compromising the viral fitness (Wei *et al.*, 2003). Certain critical neutralizing antibody epitopes are buried within the trimeric structure of gp120 and masked by variable regions such as V1 and V2, and can only be exposed during the conformational changes triggered by gp120-cellular receptor binding (Kwong *et al.*, 1998; Wyatt *et al.*, 1998).

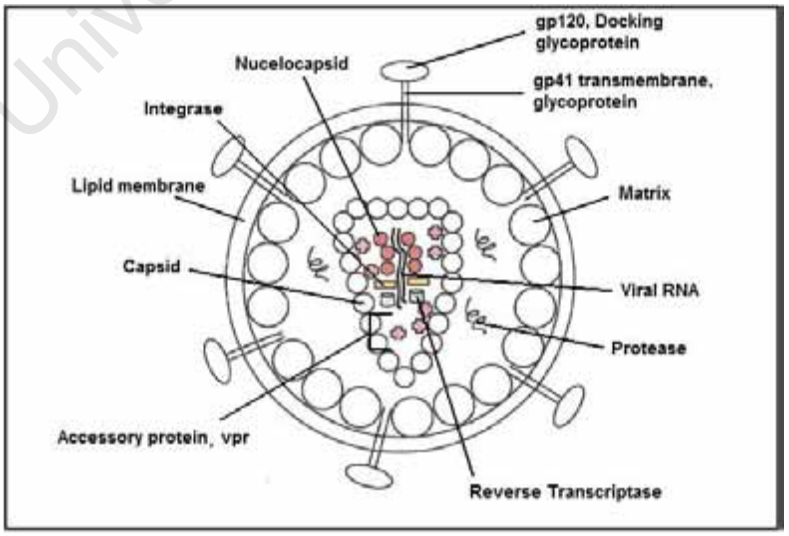

Figure 1.4 Schematic representation of the HIV-1 virion. (Giri *et al.*, 2004)

Gp120 consists of variable regions such as V1-V5, which form loops on the surface of the glycoprotein and shield the conserved core region of gp120 (Kwong *et al.*, 1998). These hypervariable regions direct the host antibody responses to these regions, so allowing escape-mutations to occur without compromising viral fitness (Burton *et al.*, 2004).

HIV-1 specific CTL responses have been shown to correlate with successful HIV-1 replication control during acute and chronic phase of the infection. However, because of the lack of proof reading ability of the RT of HIV-1, mutations are generated at a remarkably high rate of approximately 3 x 10^{-5}/ nucleotide bases per cycle of replication (Mansky & Temin, 1995). The fast-mutating HIV-1 genome enables the virus to escape the CTL responses. The HIV-1 specific CTL response creates a selection pressure favouring mutated virus variants that could escape the existing CTL response without compromising the viral fitness significantly. These variants exhibit mutations that are involved in alteration of antigen processing and presentation of the CTL epitopes which leads to the inability of CD8+ T cells to recognize these epitopes presented on infected cells (Tripathi & Agrawal, 2007). This selection pressure for CTL escape variants is further supported by the more frequent occurrence of mutations within the CTL epitopes (Moore *et al.*, 2002b).

HIV-1 also evades immune control by modulating the immune components of the host via HIV-1 proteins such as Vpr, Env and p24 (Luzzati *et al.*, 1994; Piguet *et al.*, 1999). The most well investigated mechanism is that of immunosuppression mediated through HIV-1 Nef. HIV-1 Nef protein has been documented to downregulate the expression of surface molecules such as CD4, CD28 and MHC class I on the surface of T lymphocytes and antigen presenting cells (Atkins *et al.*, 2008; Swigut *et al.*, 2001; Yang *et al.*, 2002). The downregulation of these surface molecules affects the interaction of the antigen presenting cell with T lymphocytes and enables the virus to evade HIV-1 specific immune control.

During the early course of HIV-1 infection, CD4+ T cell depletion in the gut could result in dysfunction of the cellular and humoral responses and prevent effective HIV-1 specific immune responses being induced (Fukazawa *et al.*, 2008; Guadalupe *et al.*, 2003).

These evasive mechanisms exhibited by HIV-1 create great obstacles for vaccine development and seriously compromise the effectiveness of vaccination.

1.1.3.3 HIV-1 latency and the short window of opportunity for vaccine elicited control

The HIV-1 Pol gene codes for several viral enzymes, namely, protease (PR), reverse

transcriptase (RT) and integrase. RT reverse transcribes the HIV-1 RNA genome into double stranded DNA (Gotte *et al.*, 1999; Jonckheere *et al.*, 2000; Miller *et al.*, 1997) . The HIV-1 DNA is then incorporated into the human genome through integrase activity (Miller *et al.*, 1997). The ability of HIV-1 to integrate into the genomic DNA of infected resting CD4+ T cells, enables the provirus to remain latent and undetected from immune surveillance of CTL responses. A latent reservoir of provirus can be established in resting CD4+ T cells (Chun *et al.*, 1998) and complete eradication of the virus can not be achieved, even with extensive antiretroviral therapy which reduces plasma viral load to an undetectable level (Siliciano *et al.*, 2003). HIV-1 establishes this latent reservoir in the early course of the infection (Chun *et al.*, 1998). Therefore the window of opportunity for vaccine induced HIV-1 specific immunity to prevent HIV-1 infection is relatively short, within hours to days post infection (Chun *et al.*, 1998; Johnston & Fauci, 2007). This further complicates vaccine development, as the ideal HIV-1 vaccine would have to induce a systemic and mucosal memory response that could be recalled within this short period of time to prevent the establishment of a HIV-1 latent reservoir.

1.1.3.4 Inadequate immune correlates of protection

The precise mechanisms involved in the control of HIV-1 replication in certain individuals and the types of responses needed to be induced by vaccines to achieve this control has not been completely defined to date. Information on the immune correlates of protection against HIV-1 infection is critical for the design of HIV-1 vaccines and the development of immunological assays to accurately predict vaccination outcome. The lack of this information has seriously delayed the development of an efficacious HIV-1 vaccine.

The present data available on possible immune correlates of protective responses against HIV-1 infections was obtained from studies on elite controllers (Miura *et al.*, 2009; Pereyra *et al.*, 2008; Walker, 2007a; Walker, 2007b) and rhesus macaques, which were protected from SIV infection by live attenuated SIV vaccination (Genesca *et al.*, 2008; Jia *et al.*, 2009; Reynolds *et al.*, 2008; Whitney & Ruprecht, 2004; Yankee *et al.*, 2009) as mentioned in section 1.1.2.

With further elucidation of the immune mechanisms involved in the protective efficacy observed in the RV144 trial (Rerks-Ngarm *et al.*, 2009), critical questions such as relevant immune correlates and the effectiveness of the prime-boost vaccine regimen can be answered and used to improve future HIV-1 vaccine designs. Presently observations such as the absence of significant difference in HIV-1 viremia and CD4+ T cell preservation between the vaccinated and placebo group may suggest that the immune correlate of protection may not be the same as the immune response responsible for reduced acute viraemia level (mainly CTL). The common antigen in

the priming and boosting vaccinations in the trial was envelope protein gp120. This may suggest that humoral immune response may be the immune correlate responsible for the moderate protection efficacy observed in the RV144 trial (Rerks-Ngarm *et al.*, 2009).

1.1.4 HIV-1 vaccine research and delivery strategies

1.1.4.1 T-cell and B-cell vaccines

HIV-1 vaccines are generally designed to elicit either a B- or a T-cell immune response, or a combination of both. It is commonly recognized that both arms of the immune response are essential for achieving optimum protection through vaccination against HIV-1 infection (Gauduin *et al.*, 1997; Humbert *et al.*, 2008; Jin *et al.*, 1999; Mascola *et al.*, 2005; Pantaleo *et al.*, 1995; Parren *et al.*, 2001; Rowland-Jones *et al.*, 1998; Schmitz *et al.*, 1999). B-cell vaccines usually carry envelope proteins as the vaccine antigen in order to induce neutralizing antibodies whereas T-cell vaccines usually carry HIV-1 Gag and RT or other regulatory proteins against which a cell mediated immune response can be elicited.

1.1.4.1.1 B-cell vaccines

The ability to elicit broadly neutralizing antibodies that could neutralise a broad spectrum of HIV-1 isolates remains one of the major obstacles in B-cell vaccine research (Flynn *et al.*, 2005; Pitisuttithum *et al.*, 2006). This is due to the HIV-1 hypervariability in its Env region and its immune evasive mechanisms against host neutralizing antibody responses mentioned in 1.1.3.1 and 1.1.3.2. However, broadly neutralizing antibodies still exist *in vivo* in HIV-1 infected patients (Burton *et al.*, 1991; Ferrantelli *et al.*, 2004b; Pantaleo *et al.*, 1995). Amongst these, only a small percentage of neutralizing antibodies alone or in combination, can neutralize virus isolates from different clades *in vitro* (Binley *et al.*, 2004; Ferrantelli *et al.*, 2004a; Kitabwalla *et al.*, 2003; Walker *et al.*, 2009; Xu *et al.*, 2001). Nevertheless, this observation still provides support for the possibility of eliciting broadly neutralizing antibody by vaccination.

Passive immunization of broadly neutralizing antibodies in macaques provided complete protection against virulent SHIV challenges (Ferrantelli *et al.*, 2004b; Ferrantelli *et al.*, 2004a; Mascola *et al.*, 1999; Parren *et al.*, 2001; Veazey *et al.*, 2003). These results support the importance of neutralizing antibodies to achieve complete protection against HIV-1 infection and the possibility of discovering the protective epitopes by studying the targets of these broadly neutralizing antibodies. The interactions between these antibodies and their target HIV-1 epitopes have provided important information that could improve antigen design for future HIV-1 vaccines. These epitopes are usually located at conserved regions such as the receptor binding

sites on gp120 and gp41. These include CD4 binding sites on gp120 recognized by neutralizing antibody b12 and F105 (Posner *et al.*, 1991; Saphire *et al.*, 2001), mannose residue on gp120 recognized by neutralizing antibody 2G12 (Calarese *et al.*, 2005) and membrane proximal external region of gp41 recognized by neutralizing antibodies 4E10 and 2F5 (Cardoso *et al.*, 2005; Zwick *et al.*, 2005). Functional constraints on these epitopes for receptor binding, may prevent hypervariability in these regions. However, some of these epitopes are hidden in their normal protein configuration and are only transiently available after structural change during binding and fusion to the target cells. This feature allows the virus to avoid immune surveillance by the host (Kwong *et al.*, 1998; Wei *et al.*, 2003; Wyatt *et al.*, 1998). Some neutralizing antibodies have been demonstrated to be autoreactive to host protein such as cardiolipin (Haynes *et al.*, 2005). These observations may explain why HIV-1 specific neutralizing antibody responses are not easily generated, neither through infection nor vaccination.

Different approaches have been investigated to try and improve the ability of B-cell vaccines to elicit broadly neutralizing antibody responses. One of the approaches was to investigate the protective epitopes targeted by broadly neutralizing antibodies. The technique of phage display peptide libraries for the production of mimotopes that mimic HIV-1 env epitopes, has been used as a high throughput screening method for elucidating the protective epitopes targeted by neutralizing antibodies (Humbert *et al.*, 2008). Mice primed with a DNA vaccine encoding HIV-1 gp160 and boosted with phage expressing mimotopes targeted by neutralizing antibodies, induced neutralizing antibodies and can neutralize both HIV-1 subtype B and subtype C isolates (Humbert *et al.*, 2008). Another approach involves the generation of modified HIV-1 surface molecules that are stable intermediate complexes that would expose the normally concealed epitopes such as the CD4 binding sites on gp120 for antibody binding (Banerjee *et al.*, 2009; Bontjer *et al.*, 2009; Douek *et al.*, 2006; Wu *et al.*, 2009) .

A role for non-neutralizing antibodies in the protective response against HIV-1 has also been suggested (Hessell *et al.*, 2007). Despite the absence of neutralizing activity against HIV-1 isolates in *in vitro* neutralization assays, the Fc region of these antibodies are required for the antiviral responses against HIV-1, mediated through complement binding to Fc receptors to induce lysis or phagocytosis of antibody-bound viruses (Banki *et al.*, 2005; Huber *et al.*, 2006; Huber *et al.*, 2008; Willey & asa-Chapman, 2008).

1.1.4.1.2 T-cell vaccines

The failure in HIV-1 vaccine research thus far to produce a successful B-cell vaccine that can elicit HIV-1 specific broadly neutralising antibodies (Flynn *et al.*, 2005; Pitisuttithum *et al.*, 2006), and the evidence supporting the importance of CTL responses in the control of HIV-1

viral load and disease progression in elite controllers (Buchbinder *et al.*, 1994; Pereyra *et al.*, 2008), has provided the rationale for developing HIV-1 T-cell vaccines that are designed to induce effective HIV-1 specific cellular immunity. T-cell vaccines may not be able to prevent HIV-1 infection upon exposure, but ideally would be able to induce effective immune responses to control the dissemination of HIV-1 and delay the disease progression of HIV-1 infected individuals to AIDS. A decrease of only half a log of viral RNA results in a significant delay in disease progression (Gupta *et al.*, 2007). Even though T-cell vaccines may not prevent HIV-1 infection they should reduce the secondary transmission of HIV-1 to uninfected individuals. The probability of HIV-1 secondary transmission is directly related to the chronic viral load level in infected individuals. Studies have shown that sexual transmission from HIV-1 infected individuals to their HIV-1 negative partners is highly inefficient when their chronic plasma viral load is below 1700 copies of viral RNA/ml (Gray *et al.*, 2001; Quinn *et al.*, 2000). A similar observation was made in the case of vertical transmission from mother to child. Vertical transmission failed when the mother's plasma viral load was under 1000 viral RNA copies/ml (Garcia *et al.*, 1999). Therefore an HIV-1 T-cell vaccine that could elicit effective immune control over acute and chronic viraemia would not only benefit the infected individuals by delaying disease progression and the use of antiretroviral treatment, it would also have an impact on the overall epidemic by limiting secondary transmission, even when the vaccine does not provide sterile protection against HIV-1 infection.

There are many strategies that have been investigated for developing HIV-1 T-cell vaccines. These vaccine candidates have provided partial protection and control of viral replication in animal models (Barouch *et al.*, 2000; Casimiro *et al.*, 2005; Reynolds *et al.*, 2008; Shiver *et al.*, 2002; Wilson *et al.*, 2006). The combination of a DNA vaccine expressing SIV Gag and HIV-1 Env and a fusion protein consisting of IL2 and Fc portion of immunoglobulin, did not protect the macaques from pathogenic SHIV89.6P infection, but was able to induce potent CTL responses and reduce the viral set point and CD4+ T cell loss (Barouch *et al.*, 2000). A prime and boost combination with DNA and Adenovirus type 5 expressing SIV Gag, induced Gag-specific cellular immunity, which was correlated to the short term control and reduction in viral load in these macaques after SIV challenge (Casimiro *et al.*, 2005). Macaques vaccinated with attenuated SIV were able to reduce the initial viral replication by a level of 2 logs post challenge with a pathogenic heterologous strain of SIV. Furthermore, the transient depletion of CD8+ T cells resulted in an increase in SIV replication, demonstrating that vaccine induced immune control on SIV replication is CD8+ T cell related (Reynolds *et al.*, 2008). The Merck adenovirus type 5 vaccine expressing HIV-1 Gag, Nef and pol was the first T-cell vaccine candidate to be evaluated in a human phase IIb trial (STEP) (Corey *et al.*, 2009; McElrath *et al.*, 2008).

However the trial was terminated due to the lack of efficacy in vaccinated individuals. There were no significant differences in the level of plasma viral level between the vaccinated group and the placebo control group. The vaccination also resulted in a higher risk of HIV-1 infection in individuals that were positive for adenovirus type 5 before immunization, compared to the placebo control group (Corey *et al.*, 2009; McElrath *et al.*, 2008). Although the outcome of the trial was disappointing, it does not rule out the possibility of the development of a successful T-cell vaccine.

Although the cellular immune correlation to the control of HIV-1 replication remains unclear, there are several observations made in elite controllers that could provide clues for future T-cell vaccine designs. Elite controllers exhibit increased frequency of polyfunctional HIV-1 specific CD4+ and CD8+ T cells compared to progressors. These individuals have CD4+ and CD8+ T cells that secrete multiple cytokines and chemokines, and degranulate simultaneously (Almeida *et al.*, 2007; Betts *et al.*, 2006; Harari *et al.*, 2007; Pereyra *et al.*, 2008). The polyfunctionality of the T cells are thought to be important for viremic control (Makedonas & Betts, 2006). CTL of elite controllers preferentially target conserved epitopes in HIV-1 Gag, (Goepfert *et al.*, 2008; Makedonas & Betts, 2006; Miura *et al.*, 2009; Pereyra *et al.*, 2008; Streeck *et al.*, 2007; Wang *et al.*, 2009); however, other conserved epitopes in Nef and Pol are also associated with protective HLA allele restrictions (Wang *et al.*, 2009).The level of protection observed in these controllers was thought to be attributed to the strong CTL responses specific to escape variants, which have compromised viral fitness. Lower magnitude and fewer targeted epitopes of the HIV-1 specific responses were observed in elite controllers compared to progressors (Pereyra *et al.*, 2008), indicating that a high magnitude of HIV-1 specific CTL response is not essential for the control of acute HIV-1 replication (Masemola *et al.*, 2004; Pereyra *et al.*, 2008). The above results suggest that an ideal HIV-1 T cell vaccine should elicit polyfunctional CTL responses specific to conserved epitopes in Gag, although other conserved epitopes in Pol and Nef should also be included in the vaccine to cover the HLA allele diversity in the population (Wang *et al.*, 2009).

1.1.4.2 Different vaccine delivery systems

The protection provided by live attenuated SIV vaccination in NHPs against homologous or heterologous strains of SIV challenge has been demonstrated to be the most promising vaccination approach in NHPs thus far (Evans *et al.*, 2005; Jia *et al.*, 2009; Wyand *et al.*, 1996; Wyand *et al.*, 1999). However this approach is unlikely to be adopted as a plausible strategy for HIV-1 vaccination due to the safety concern of the attenuated HIV-1 reverting to a virulent strain, which subsequently can cause persistent infection and disease in vaccinated individuals, as has been demonstrated in the attenuated SIVmac239, where recombination occurred between the

vaccine and challenge strains of SIV in macaques (Reynolds *et al.*, 2008) . Other safer delivery systems have been evaluated for the delivery of HIV-1 antigens to induce HIV-1 specific immune responses. The different vaccine delivery systems have been extensively reviewed by (Girard *et al.*, 2006) and (Giri *et al.*, 2004). These include DNA vaccines, Virus-like particles (VLPs) (Chege *et al.*, 2008; Chen *et al.*, 2005; Doan *et al.*, 2005; Hammonds *et al.*, 2005; Harvey *et al.*, 2003; Zhang *et al.*, 2004), subunit or protein vaccines (Banerjee *et al.*, 2009; Bontjer *et al.*, 2009; Douek *et al.*, 2006; Wu *et al.*, 2009), and bacterial (Ami *et al.*, 2005; DeVico *et al.*, 2002; Someya *et al.*, 2005) and viral vector based vaccines. For the purpose of this study, the emphasis is on DNA vaccines and recombinant viral vaccines, in particular, poxviruses.

HIV-1 DNA vaccines are non-infectious DNA plasmid vectors expressing HIV-1 antigens. They cannot replicate or produce additional undesirable proteins in vaccinated individuals, thus providing a better safety profile compared to viral and bacterial vectors. DNA vaccines are known to induce both humoral and cellular immune responses (Boyer *et al.*, 1999; Boyer *et al.*, 2000; MacGregor *et al.*, 2000). Early trials showed DNA vaccines induced only modest immunogenicity (Lu *et al.*, 2008; MacGregor *et al.*, 1998; Wang *et al.*, 1998). A DNA vaccine expressing HIV-1 Env and Rev, was evaluated in a small group of asymptomatic HIV-1 infected patients as a therapeutic vaccine. The vaccination did not have any effect on the plasma viral load and CD4+ T cell count in these patients and only induced a small percentage of vaccine elicited env-specific humoral or CTL responses (MacGregor *et al.*, 1998; MacGregor *et al.*, 2000). Similar results were observed when DNA vaccination was tested in animal models. A DNA vaccine expressing HIV-1 env, only induced a transient level of Env-specific antibodies in mice (Lu *et al.*, 1995). The limited immune response induced by DNA vaccines may be attributed to the inefficiency of DNA vaccine uptake or to the low level of antigen expression from DNA vectors (Giri *et al.*, 2004). Increased levels of antigen expression from DNA vaccines have been achieved by codon-optimising the antigen gene sequence, using codons commonly used by the human transcriptional system (Andre *et al.*, 1998; Barouch *et al.*, 2000; Huang *et al.*, 2001; Van Harmelen *et al.*, 2003; Williamson *et al.*, 2003; zur Megede J. *et al.*, 2000).

The co-expression of immune modulatory molecules, which act as a molecular adjuvant, has improved the immunogenicity of DNA vaccines (Boyer *et al.*, 2005; Moore *et al.*, 2002a; Nayak *et al.*, 2006; Qiu *et al.*, 2007; Xu *et al.*, 2008). These molecular adjuvants include IL-2, IL-12, IL-15 (Xin *et al.*, 1999; Xu *et al.*, 2008), granulocyte-macrophage colony stimulating factor (GMCSF) ((Moore *et al.*, 2002a; Qiu *et al.*, 2007; Xu *et al.*, 2008) and fms-like tyrosine kinase 3 ligand (Flt-3L) (Nayak *et al.*, 2006; Xu *et al.*, 2008). Co-administration of DNA vaccine

encoding HIV-1 Env or Gag with IL-12, GMCSF or Flt-3L induced enhanced immunogenicity to these HIV-1 antigens in mice. The percentage of polyfunctional CD8+ T cells was also increased when DNA vaccines were co-administered with GMCSF (Xu *et al.*, 2008).

The uptake of DNA can be improved using more efficient means of delivery (Babiuk *et al.*, 2002a; Babiuk *et al.*, 2002b; Luckay *et al.*, 2007; Tollefsen *et al.*, 2002; Widera *et al.*, 2000). DNA vaccine was conventionally administered intramuscularly by needle injection or intradermally using a gene gun. More recently, *in vivo* electroporation of the DNA vaccine into macaques has been demonstrated to induce 10-40 times enhancement in HIV-1 specific cell mediated immune responses compared to conventional needle administration (Luckay *et al.*, 2007).

Viral vectors have been evaluated as vaccine delivery vehicles for HIV-1 antigens. The advantage of viral vectors over subunit vaccines is the ability of viral vectors to express HIV-1 antigens intracellularly, where they can be processed and presented through the MHC-class I pathway to induce HIV-1 specific CTL responses. Viral vectors are also known to induce humoral immune responses to the foreign antigen expressed. Recombinant Adenovirus serotype 5 (Ad5), has been the most immunogenic vector compared to DNA or recombinant poxvirus vaccines (Casimiro *et al.*, 2003; Santra *et al.*, 2005). However, the immunogenicity in preclinical and phase I studies did not translate to vaccine efficacy with respect to the reduction of HIV-1 acquisition in the phase II trial (Buchbinder *et al.*, 2008; Santra *et al.*, 2005). Although the vaccination regimen was immunogenic in humans and induced HIV-1 specific CD8+ T cells in most of the vaccinees (Buchbinder *et al.*, 2008; McElrath *et al.*, 2008; Santra *et al.*, 2005) the vaccination failed to induce a significant reduction in HIV-1 infection. The observation of a higher risk of HIV-1 acquisition in the vaccinated population with high pre-immunity to Ad5 compared to the placebo control group has raised concerns regarding the effect of vector pre-immunity. Rare serotypes of human Adenovirus such as Ad35 and Ad11 (Lemckert *et al.*, 2005; Lemckert *et al.*, 2006; Liu *et al.*, 2008), and other viral vectors such as poxviruses (discussed in 1.2.2), are being evaluated to try and overcome the limitation of vector pre-immunity.

There are many different HIV-1 vaccination strategies, all of which, when used individually, induce modest and short term HIV-1 specific immune responses. It is commonly accepted that heterologous prime-boost combinations using serologically-distinct vectors induce enhanced immune responses compared to single vaccination regimens and repeated inoculations using homologous vaccine vectors. The use of different vectors for priming and boosting, such as DNA prime, viral vector or protein boost, has been shown to be immunogenic in animal models

and human clinical trials (Lu, 2009; Ranasinghe & Ramshaw, 2009). Phase I clinical evaluation of a Gag expressing DNA prime, MVA boost regimen demonstrated that Gag-specific polyfunctional T cell responses were induced with the regimen in human subjects. Higher percentages of responders were observed in the group vaccinated with DNA prime, MVA boost regimen compared to the homologous MVA prime/boost regimen (Goonetilleke *et al.*, 2006). Similar immunogenicity results were observed when the DNA prime/ MVA boost regimen was evaluated in animal models (Abaitua *et al.*, 2006; Amara *et al.*, 2002; Brave *et al.*, 2007; Burgers *et al.*, 2009; Gherardi *et al.*, 2004; Hanke *et al.*, 2007; Smith *et al.*, 2004). The DNA prime/ adenovirus type 5 boost vaccination regimen could be a potential solution to overcome the problem of the high percentage of adenovirus type 5 pre-immunity in the population. The efficacy of the regimen was demonstrated in NHPs, where rhesus macaques vaccinated with a SIV Gag- expressing DNA prime, followed by an adenovirus type 5 boost regimen, were able to induce SIV Gag-specific immunity and reduce the viral load after pathogenic SIV challenge (Casimiro *et al.*, 2005). The heterologous prime/boost regimen was more efficacious in the level of protection against pathogenic SIV, compared to the homologous prime/boost with adenovirus type 5 (Casimiro *et al.*, 2005; Cox *et al.*, 2008). Analysis of the T cell profiles induced by the two regimens showed differences in the diversity of the type of T cell responses and IL-2 secretion. The DNA prime/ adenovirus boost regimen elicited a more diverse phenotype of T cell responses compared to the homologous prime/boost regimen with adenovirus type 5 (Casimiro *et al.*, 2005; Cox *et al.*, 2008). The combination of recombinant canarypoxvirus prime and gp120 protein boost has recently been evaluated in a phase III clinical trial in Thailand (Pitisuttithum *et al.*, 2006). This regimen induced HIV-1 specific antibody mediated cellular toxicity in the majority of individuals in the vaccinated group, and not in the placebo control group (Karnasuta *et al.*, 2005). Preliminary results from the trial have shown that the prime-boost vaccination regimen induced mild protection efficacy against HIV-1 infection (31% reduction in infection in the vaccinated group compared to the group that received placebo) (Berkhout & Paxton, 2009). The regimen did not induce a reduction in viral load in HIV-1 infected individuals in the vaccinated group (Berkhout & Paxton, 2009).

The heterologous prime/boost vaccination regimens are not limited to distinct classes of vectors, such as poxviruses or adenoviruses. Similar but serologically distinct vectors can be used in heterologous prime/boost regimens. Potential advantages of a specific class of vector may be shared amongst members within the class, thus it could potentially have a positive effect on the immunogenicity induced by the regimen. The prime-boost combination of adenovirus type 35 (Ad-35) and adenovirus type 11 (Ad-11) expressing SIV Gag induced significantly enhanced Gag specific T-cells compared to homologous prime-boost inoculations with either Ad35 or

Ad11 respectively. Heterologous Prime-boost combinations using diverse poxvirus vectors such as MVA, vaccinia virus and fowlpox virus expressing HIV-1 Env and SIV Gag have induced significantly higher magnitudes of SIV immune responses before challenge, containment of viral replication after SHIV challenge and reduced levels of CD4+ T cell loss compared to homologous prime-boost inoculations using MVA in macaques (Santra *et al.*, 2007)

Interesting results were observed in mice vaccinated using a prime-boost-boost regimen with three distinct vectors (Yu *et al.*, 2008). The mice were primed with DNA expressing SIV-Gag and boosted with recombinant adenovirus, followed by recombinant Sendai virus (murine parainfluenza virus type 1), both expressing SIV Gag. The triple vaccination regimen induced potent Gag specific responses, which were stronger than other double or single vaccination regimens (Yu *et al.*, 2008).

The benefit of the heterologous prime-boost vaccination strategy has provided the motivation and rationale for the search for novel vaccine vectors that can be evaluated in such combinations.

1.2 Poxviruses

1.2.1 Life cycle

Vaccinia virus is the most well researched poxvirus due to its usefulness in the smallpox eradication campaign. For this reason it has been used as a prototype virus for poxvirus research. The fundamental knowledge of poxvirus replication, transcription and morphogenesis was obtained from studying vaccinia virus. The details of this enormous field have been described in several in-depth reviews (Broyles, 2003; Moss, 1996; Moss, 2006; Schramm & Locker, 2005; Smith *et al.*, 2002; Smith, 2007)

1.2.1.1 Virion structure and viral entry

The poxvirus virion is approximately 250 nm x 350 nm in size (Smith, 2007). There are two types of poxvirus virion, intracellular mature virus (IMV) and extracellular enveloped virus (EEV) which differ in their outer membranes. IMV is enveloped in a single layer of lipid membrane (Dales & Siminovitch, 1961; Hollinshead *et al.*, 1999), while EEV has an extra lipid membrane compared to IMV (Schmelz *et al.*, 1994; Tooze *et al.*, 1993). The core of the virion is surrounded by an 18 nm porous palisade layer (Hollinshead *et al.*, 1999). Structural proteins, a double-stranded viral DNA genome, and enzymes required for early transcription are enclosed within the core (Moss, 1996; Munyon *et al.*, 1967).

Poxvirus entry begins with attachment to the cell (Figure 1.5). The attachment of the virus is facilitated by transmembrane proteins on the viral membrane of IMV. Several viral proteins and cellular receptors have been implicated in virus attachment to the cells; such as poxvirus A27 (Hsiao *et al.*, 1998), H3 (Lin *et al.*, 2000), D8 (Hsiao *et al.*, 1999) and A26 (Chiu *et al.*, 2007) which interact with cell surface glycosaminoglycan (GAG), heparan sulfate proteoglycan (HSPG), chondroitin sulfate proteoglycan (CSPG), and extracellular matrix protein laminin respectively. However none of these viral-cell protein interactions alone is essential for virus attachment to the cells (Chiu *et al.*, 2007; Hsiao *et al.*, 1998; Hsiao *et al.*, 1999; Rodriguez & Smith, 1990). Possible alternative mechanisms could still allow attachment to take place.

Poxvirus core entry into the cell is achieved through fusion of the IMV membrane and the cell plasma membrane (Figure 1.5). The fusion between the two membranes is facilitated by a multi-viral protein entry/fusion complex (EFC), consisting of A21, A28, H2, L5, A16, G3, G9, and J5 (Senkevich *et al.*, 2005). A21, A28, G3, H2 and L5 are proteins with N-terminal transmembrane domains, while J5, A16 and G9 are proteins with C-terminal transmembrane domains (Senkevich *et al.*, 2005). Null mutant vaccinia viruses with all of the above genes interrupted/deleted were still able to attach to the target cells, but not enter the cells, suggesting a block in fusion between the viral and cell plasma membrane (Moss, 2006; Senkevich & Moss, 2005). Recent studies revealed that L1 and F9 proteins are also essential for the fusion process. Like the EFC suppressed mutant vaccinia virus, L1 or F9 suppressed vaccinia mutants were able to attach to the target cells but unable to enter the cell (Brown *et al.*, 2006a; Foo *et al.*, 2009). The importance of these viral proteins in the poxvirus life cycle is evident from the conservation of these genes across the different genera of poxviruses (Moss, 2006; Senkevich *et al.*, 2005).

Because EEV has an extra membrane compared to IMV, additional mechanisms to disrupt the external membrane are required before viral entry can take place (Figure 1.5). The disruption of the outer membrane of EEV is achieved through a ligand-induced, non-fusogenic mechanism, where the interaction of EEV surface proteins A34 and B5 to polyanionic glycosaminoglycans on the cell plasma membrane, leads to the rupture of the EEV outer membrane (Law *et al.*, 2006) and exposes the IMV for entry through membrane fusion as described above. Even after its disruption, the outer membrane of EEV still covers the IMV and shields it from IMV-specific neutralizing antibodies (Smith, 2007).

After fusion of the IMV membrane with the target cell membrane, the core of the poxvirus is introduced into the cytoplasm of the target cell and transported on microtubules to the perinuclear region of the cytoplasm (Carter *et al.*, 2003) (Figure 1.5).

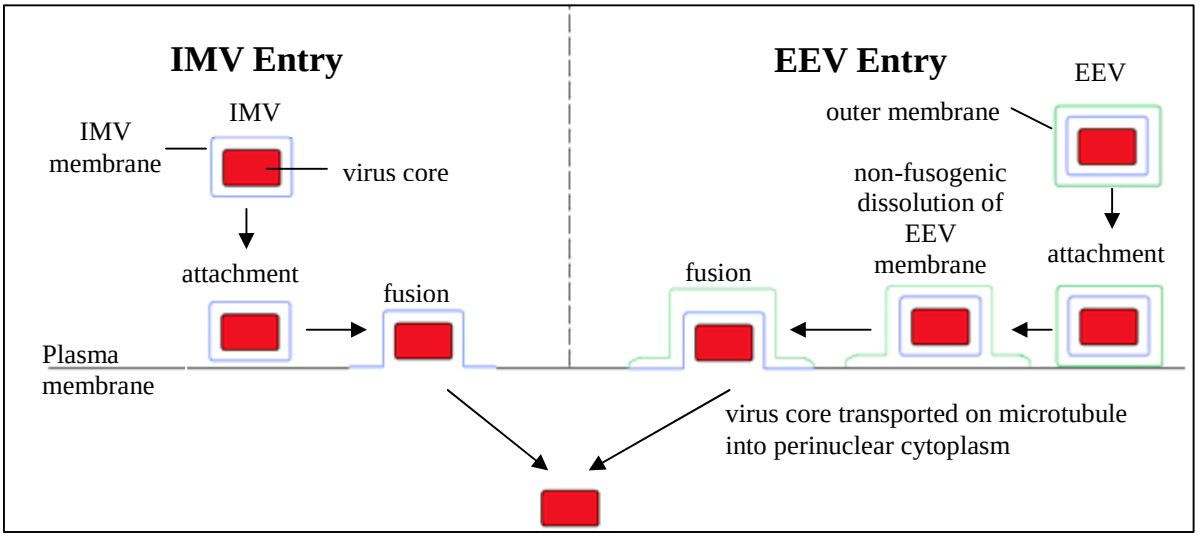

Figure 1.5 Poxvirus intracellular mature virus (IMV) and extracellular enveloped virus (EEV) entry mechanisms. IMV attachment to the cell plasma membrane receptors facilitates the fusion of IMV and cellular membranes and enables entry of the virus core into the cytoplasm. The disruption of the outer membrane on EEV is mediated through glycosaminoglycan. The disruption of the outer membrane releases the internal IMV to enter the cell cytoplasm as described above. The entered virus core is transported on microtubules to a perinuclear replication site. The diagram is adapted from that published by (Smith, 2007).

1.2.1.2 Poxvirus replication, transcription and morphogenesis

The two strands of the poxvirus's double stranded DNA genome are covalently linked by terminal hair-pin loop structures into a single DNA molecule (Baroudy *et al.*, 1982; Berns & Silverman, 1970). Inverted terminal repeats (ITR) are located adjacent to the hair-pin loops (Goebel *et al.*, 1990). The length of these repeated sequences varies between different poxviruses from <0.1kb to 12.4kb,(Wittek *et al.*, 1978). Within the ITR, shorter tandemly repeated sequences and a highly conserved concatemer resolution motif can be found (Merchlinsky, 1990; Merchlinsky & Moss, 1989). The motif is essential for the resolution of concatemer DNA molecules during poxvirus DNA replication (Merchlinsky, 1990; Merchlinsky & Moss, 1989).

The poxvirus genome contains open reading frames that are closely packed with little non-coding intragenic sequences (Goebel *et al.*, 1990). The central region of the poxvirus genome is relatively conserved within the different genera (Gubser *et al.*, 2004) and higher variability occurs in the terminal regions of the virus genome (Gubser *et al.*, 2004). Poxvirus genes can be divided into three groups according to their time of expression in the virus life cycle - early, intermediate or late (Broyles, 2003). The components required for early transcription, such as viral DNA-dependent RNA polymerase, capping enzyme, polyA-polymerase and early transcriptional initiation and termination factors are packed inside the viral core (Kates & McAuslan, 1967; Shuman *et al.*, 1987; Wei & Moss, 1974; Yuen *et al.*, 1987). The presence of the early transcriptional system allows poxvirus early genes to be independently transcribed of the host, immediately after penetration of the core into the cytoplasm of the host cell. These

early genes code for proteins that are involved in viral DNA synthesis and defence against the host immune response (Moss, 1996), and are under the control of early poxvirus promoters that can only be recognized by poxvirus RNA polymerase (Davison & Moss, 1989).

Replication of Poxvirus DNA occurs within 2 hours post-infection (Salzman, 1960). It takes place after early gene expression and uncoating of the viral core in cytoplasmic viral factories (Cairns, 1960). DNA replication begins with a nick in one of the DNA strands near the terminal hair-pin loop. This opens up the linked double strands and allows for self priming and elongation of the viral DNA along the whole genome by a poxviral DNA polymerase. A concatameric replicative intermediate is formed which, when nicked twice, is resolves into two monomeric genomes (Eckert *et al.*, 2005; Shuman & Moss, 1987). Parallel to DNA replication, intermediate genes are expressed under the control of functionally distinct intermediate promoters (Baldick, Jr. *et al.*, 1992). These intermediate genes, A1L, A2L and G8R encode for transcriptional factors required for late transcription (Keck *et al.*, 1990; Kovacs *et al.*, 1994; Wright & Coroneos, 1993)

Late genes generally encode structural proteins for virion formation. Some of the late genes also encode the enzymes and transcriptional factors required for early gene transcription mentioned previously. Expression of the late genes are under the control of poxvirus late promoters (Rosel *et al.*, 1986). The initiation of poxvirus late transcription is dependent on poxvirus DNA replication and the expression of late transcription factors encoded by the intermediate genes, early gene H5L (Kovacs *et al.*, 1994; Kovacs & Moss, 1996)and a host factor VLTF-X (Gunasinghe *et al.*, 1998; Wright *et al.*, 1998).

After DNA replication and late gene expression, viral crescents, which are composed of viral protein associated with a host derived single layer lipid membrane, are formed within viral factories (Dales & Siminovitch, 1961) (Figure 1.6). The poxvirus DNA genome and viral enzymes are packed into the viral crescents to form immature virions (IV), which mature into IMV through proteolysis of the core capsid proteins (Moss & Rosenblum, 1973). IMV forms the majority of the infectious poxvirus progeny and is released through lysis of infected cells (Smith *et al.*, 2002). Some IMV particles are transported away from the viral factories through microtubules (Sanderson *et al.*, 2000), and are further processed through the trans-Golgi or endosome, where they acquire an additional double-layered intracellular membrane and are know as intracellular enveloped virus (IEV) (Hiller & Weber, 1985; Schmelz *et al.*, 1994) . IEV is transported through microtubules to the plasma membrane of the cell (Hollinshead *et al.*, 2001). The fusion of the cell plasma membrane and the outer membrane surrounding the IEV

allows for export of IEV from the surface of the cell by exocytosis (Smith, 2007). The virion can either remain attached to the cell (cell-associated enveloped virus, CEV) or be released as EEV. The association of CEV with the cell induces actin tail formation which is important for cell to cell spread of the virus (Frischknecht *et al.*, 1999b; Frischknecht *et al.*, 1999a; Smith *et al.*, 2002) (Figure 1.6).

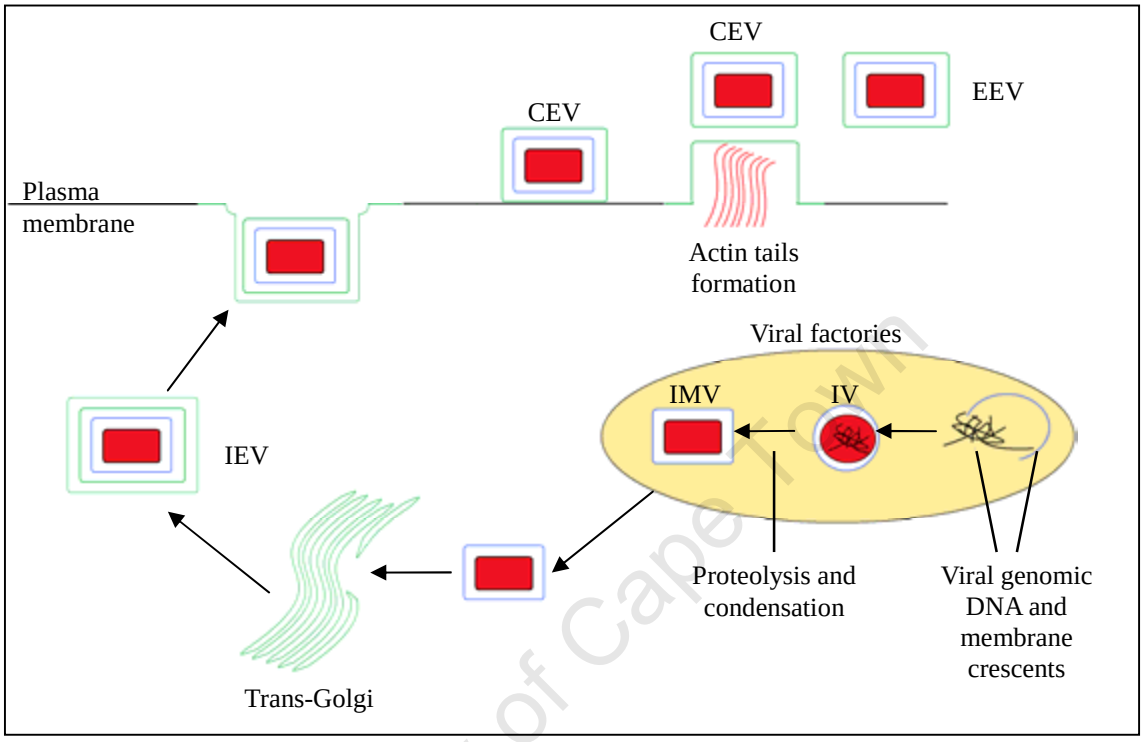

Figure 1.6 Poxvirus morphogenesis pathways. Viral DNA, proteins and crescent membranes are synthesized in the cytoplasmic viral factories. Viral DNA and protein are then packed with crescent membranes to form the immature virus (IV). IV is then matured into intracellular mature virus (IMV) through proteolytic cleavage of the capsid proteins and condensation. IMV is transported out of the viral factories on microtubules and is wrapped with a double layered plasma membrane as it passes through the trans-Golgi network to form the intracellular enveloped virus (IEV). IEV is transported to the cell plasma membrane and exits the cell surface by exocytosis. The virus can stay associated with the cell as cell-associated enveloped virus (CEV) or released from the cell as extracellular enveloped virus (EEV). CEV may induce actin tail formation in the cell cytoplasm that can aid the cell to cell transmission of the virus. This figure is adapted from that published by (Smith, 2007).

1.2.2 Poxviruses as vaccine vectors

The successful eradication of smallpox in 1980 through vaccinia virus (VV) vaccination (WHO, 1980) provided evidence of the effectiveness of the eradication of an infectious disease through vaccination. The success of vaccinia virus as a vaccine, along with the development of DNA modification techniques for this virus, has led to the realization of the potential of poxviruses as vaccine vectors for other infectious agents (Mackett *et al.*, 1982; Mackett *et al.*, 1984; WHO, 1980). Vaccinia virus has several desirable properties as a vaccine vector. It can induce both cellular and humoral immune responses (Cooney *et al.*, 1991; Cooney *et al.*, 1993; Elango *et al.*,

1986; Graham *et al.*, 1992; Nixon *et al.*, 1988). Large inserts of foreign DNA can be incorporated into the vaccinia virus genome without affecting the viral life cycle (Smith & Moss, 1983). Vaccinia virus can be used in human and veterinary vaccine applications because it has a wide host range. The lack of viral DNA integration into the host genome and viral persistence makes it a safe vector. The use of recombinant vaccinia virus expressing the immunizing G protein of rabies virus has demonstrated the effectiveness of vaccinia virus as a recombinant vaccine vector. With the oral vaccination of raccoons and foxes through bait, it has significantly reduced the incident rate of wildlife rabies occurring in European countries and the United States of America (Pastoret & Brochier, 1996; WHO, 1980).

Due to the HIV-1 pandemic, there is a significant part of the population that is or could be potentially immunocompromised. Transfer of vaccinia virus could occur from vaccinated individuals to non-vaccinated contacts. The possible dissemination of vaccinia virus in immunocompromised individuals (Redfield *et al.*, 1987) presents a great safety concern for its application as a human vaccine vector. This undesirable feature of vaccinia virus has led to the development of other safer poxvirus vectors such as highly attenuated, replication-deficient modified vaccinia Ankara (MVA) and other host-range restricted poxviruses, such as avipoxviruses and capripoxviruses (Aspden *et al.*, 2003; Baxby & Paoletti, 1992; Bejon *et al.*, 2007; de Bruyn G. *et al.*, 2004; Jentarra *et al.*, 2008; Nitayaphan *et al.*, 2004; Vijaysri *et al.*, 2008). Recently a number of studies have also evaluated several replication-competent vaccinia viruses (Copenhagen, NYCBH, Western Reserve and vaccinia Tian Tan), attenuated through deletion of virulence genes, as recombinant vaccine vectors (Huang *et al.*, 2009; Jentarra *et al.*, 2008; Vijaysri *et al.*, 2008). The efficacies of these attenuated replication-competent vaccinia viruses as vaccine vectors were demonstrated in mice (Huang *et al.*, 2009; Jentarra *et al.*, 2008; Vijaysri *et al.*, 2008) and their safety was demonstrated in immunocompromised mice (Jentarra *et al.*, 2008; Vijaysri *et al.*, 2008).

Table 1.1 Classification of poxviruses of vertebrates and the type species in each genus (Mercer *et al.*, 2007).

Subfamily :*Chordopoxviridae* Genus	 Type species
Orthopoxvirus	Vaccinia virus
Parapoxvirus	Orf virus
Avipoxvirus	Fowlpox virus
Capripoxvirus	Sheeppox virus
Leporipoxvirus	Myxoma virus
Suipox virus	Swinepox virus
Molluscipoxvirus	Molluscum contagiosum virus
Yatapoxvirus	Yaba monkey tumor virus

1.2.2.1 Modified Vaccinia Ankara (MVA)

MVA was derived from the Ankara strain of vaccinia virus. The attenuation was achieved by passaging the virus 572 times in chicken embryo fibroblasts (Meyer *et al.*, 1991), during which time approximately 30 kb DNA became deleted from the termini of the virus genome. The attenuation led to the loss of the ability of the virus to replicate in most mammalian cell lines. However MVA is able to replicate productively in the Syrian hamster kidney cell line, BHK-21 (Carroll & Moss, 1997) and, arguably, in the rat small intestinal epithelial cell line IEC-6 (Okeke *et al.*, 2006). MVA has been demonstrated to exhibit abortive replication in all other mammalian cells (Zhang *et al.*, 2007) and to be non-pathogenic in immunocompromised animals (Wyatt *et al.*, 2004). MVA was deployed as a smallpox vaccine during the later stage of the vaccination campaign in Germany and the vaccination of humans with MVA did not result in any complications (Stickl *et al.*, 1974). The superior safety profile of MVA over vaccinia virus made MVA an attractive and competitive vaccine vector against several infectious diseases. A recombinant MVA expressing *mycobacterium tuberculosis* 85A antigen (MVA85A) has been developed as a promising Tuberculosis vaccine, which has been demonstrated to be both safe and immunogenic in human clinical trials (Hawkridge *et al.*, 2008; McShane, 2009; Sander *et al.*, 2009). Several studies and clinical trials have also demonstrated the protective immunogenicity of recombinant MVA expressing the Plasmodium berghei circumsporozoite protein of the malaria parasite (Hill *et al.*, 2000; Hutchings *et al.*, 2007; Moore & Hill, 2004; Prieur *et al.*, 2004). MVA has attracted huge interest as a vaccine vector in the field of HIV-1 vaccine research (Burgers *et al.*, 2009; Greenough *et al.*, 2008; Im & Hanke, 2004; Sandstrom *et al.*, 2008), and is currently being evaluated in several clinical trials as a booster vaccine after priming with DNA (HIV Vaccine Trials Network database and International AIDS vaccine initiative trial database).

Poxviruses are known to induce long lasting anti-viral immunity in vaccinated individuals (Amara *et al.*, 2004; Hammarlund *et al.*, 2003) and a significant percentage of the world population was vaccinated with vaccinia virus during the smallpox eradication campaign. The pre-existing anti-MVA immunity in vaccinia virus vaccinated individuals, due to cross-reactivity between MVA and vaccinia virus, may reduce and hamper the effectiveness of MVA as a vaccine delivery vector in these individuals (Baxby & Paoletti, 1992; Belyakov *et al.*, 1999; Gomez *et al.*, 2008; Naito *et al.*, 2007; Nigam *et al.*, 2007). The possible large-scale deployment of MVA as a smallpox vaccine (in the event of a threat of biological warfare) or as a vaccine vector against a particular infectious agent, could lead to the presence of anti-MVA immunity in vaccinated individuals and render the vector ineffective for subsequent vaccine development against other infectious diseases. The cross-reactivity of MVA and vaccinia virus, and the

competitiveness for the use of MVA as a vaccine vector, provide the rationale for the need for additional antigenically distinct, nonpathogenic poxviruses as vaccine vectors.

1.2.2.2 Avipoxviruses

The Avipoxviruses, a genus in the subfamily *Chordopoxviridae* (Table 1.1), are host-restricted to avian hosts and replication-deficient in most mammalian cells. They have been extensively researched as veterinary and human vaccine vectors. The two most well known vectors in this genus are fowlpoxvirus and canarypoxvirus (ALVAC) (Taylor *et al.*, 1991b; Tulman *et al.*, 2004). The vaccine strains of these two poxviruses were initially evaluated and developed to vaccinate birds against avian diseases (Boyle, 2007) such as avian influenza. Vaccination of chickens with recombinant fowlpoxvirus expressing H5 hemagglutinin (HA) and N1 neuraminidase of the avian influenza virus could completely protect the birds from highly pathogenic H5N1 avian influenza virus (Qiao *et al.*, 2009; Qiao *et al.*, 2003). The immunogenicity of recombinant avipoxviruses has also been demonstrated in a wide range of non-avian species (Boyle, 2007). Recombinant Avipoxviruses have been evaluated as vaccines against rabies, canine distemper, feline leukaemia and West Nile viruses and have been demonstrated to protect against these mammalian diseases (Minke *et al.*, 2004; Pardo *et al.*, 1997; Poulet *et al.*, 2003; Taylor *et al.*, 1991b). Recombinant canarypoxvirus expressing rabies glycoprotein provided comparable levels of protection against rabies challenge in mice to that of recombinant vaccinia virus expressing the same antigen (Taylor *et al.*, 1991b). However the recombinant fowlpoxvirus expressing rabies glycoprotein elicited only partial protection (Taylor *et al.*, 1991b).

Avipoxviruses have also been explored in the search for safer and more efficacious vaccine vectors for HIV-1 vaccines. They have been shown to be immunogenic in prime/boost combinations with DNA vaccine, protein subunit vaccines and MVA (Dale *et al.*, 2004; Gupta *et al.*, 2002; Kent *et al.*, 1998; Nitayaphan *et al.*, 2004; Robinson *et al.*, 1999; Russell *et al.*, 2007; Santra *et al.*, 2007). Recombinant canarypoxvirus expressing HIV-1 subtypes B and E gp120 (ALVAC-HIV vCP1521) was deployed in prime-boost combination with gp120 protein in phase I and II clinical trials and was shown to be safe and immunogenic in HIV-1 negative individuals (Nitayaphan *et al.*, 2004). The vaccination strategy has recently been evaluated in a phase III clinical trial in Thailand. Preliminary result indicates a mild protective efficacy of 31% in the vaccinated group (Berkhout & Paxton, 2009; Rerks-Ngarm *et al.*, 2009).

Unlike MVA, the abortive steps of fowlpox virus and canarypox virus in non-permissive human cell lines occur relatively early in the virus replication cycle (Taylor *et al.*, 1995). The

avipoxviruses were only able to transcribe early genes before DNA replication of the viral genome was blocked. This early block in the virus replication process in non-permissive hosts (non-avian) may limit the level and duration of foreign antigen expression from recombinant avipoxviruses, and could be linked to the weak and limited immunogenicity induced in earlier clinical trials (Russell *et al.*, 2007).

1.2.2.3 Lumpy skin disease virus

Lumpy skin disease virus (LSDV) belongs to the *Capripoxvirus* genus of the *Poxviridae* family (Alexander *et al.*, 1957) (Table 1.1). Other members in the genus include sheeppox virus and goatpox virus (Diallo & Viljoen, 2007). LSDV is the aetiologic agent of lumpy skin disease (LSD) in cattle and was isolated from skin lesions of infected cattle in 1959 (Alexander *et al.*, 1957). The disease was first documented in Zambia in 1929 (Barnard *et al.*, 1994) after which the epidemic spread throughout southern Africa (Carn, 1993; Davies, 1991). Later, LSD spread throughout the African continent (Diallo & Viljoen, 2007). There have also been reports of LSD in the middle-east in countries such as Israel and Saudi Arabia (Greth *et al.*, 1992; Yeruham *et al.*, 1995).

The clinical symptoms of LSD include fever and the formation of necrotic skin nodules 4 to 11 days post infection (Weiss, 1968). Necrotic lesions in the respiratory tracts can lead to fatal complications in infected cattle (Weiss, 1968). The mortality rate of LSD is approximately 40% (Diallo & Viljoen, 2007; Henning, 1956). LSD can also affect milk production in infected cows, and infected bulls and cows could be permanently infertile (Diallo & Viljoen, 2007; Henning, 1956). The presence of nodules may permanently damage the hides of the cattle. These LSD related factors have a significant negative economic impact on downstream industries.

The host-range of LSDV is restricted to ruminant animals; however, the virulence of the virus appears to be restricted to cattle (Ali *et al.*, 1990; Kitching *et al.*, 1989). Infection of other ruminant animals, such as buffaloes (Ali *et al.*, 1990), which results in milder symptoms, has been documented. A low prevalence of LSDV specific antibody was also detected in serological surveys of wild-life samples and by experimental inoculation of game animals with LSDV (Davies, 1981; Young *et al.*, 1970). The experimental inoculation of sheep with LSDV resulted in a localised reaction at the site of inoculation, with no LSD-like symptoms (Kitching *et al.*, 1989). However some replication of LSDV is thought to take place as virus could be recovered from inflamed lymph nodes (Barnard *et al.*, 1994). The transmission of LSDV through contact of cattle is not efficient (Carn & Kitching, 1995). Transmission of LSDV is believed to occur via biting insects (Carn & Kitching, 1995). This hypothesis has been supported by the successful transmission of LSDV by the mosquito, *Aedes aegypti* (Chihota et al., 2001). The same

mechanical transmission of LSDV was observed for arthropod vectors (Carn & Kitching, 1995). The transmission from infected cattle to naïve cattle failed in the absence of insects (Carn & Kitching, 1995).

Because the transmission of LSDV is likely to be facilitated though biting insects, the control of flying insects could be effective in the control of a LSD outbreak. However the control and prevention of LSDV infection can also be achieved through vaccination. The LSDV vaccine was developed by passaging the Neethling strain of LSDV in embryonated eggs and attenuating it though multiple passages in lamb kidney cells and on chick chorio-allantoic membranes (CAM) (van Rooyen *et al.*, 1969). The passaging resulted in several insertions, deletions and frame shift mutations in many of the terminal variable genes and the central conserved genes (Kara *et al.*, 2003). The DNA modifications in these genes led to attenuation through impairment of DNA replication and through the destruction of many immune evasive mechanisms (Kara *et al.*, 2003). Although the vaccine can effectively protect cattle from LSDV infection, adverse effects, such as localized swelling at inoculation sites and reduction in milk production, could occur as a result of vaccination (Diallo & Viljoen, 2007). Because LSDV, sheeppox virus and goatpox virus are serologically identical (Kitching, 1986), sheeppoxvirus strains have been used as an alternative vaccine in areas such as Kenya, Egypt and Israel to control the outbreak of LSD in these regions (Davies, 1991).

Similar to vaccinia virus and avipoxviruses, LSDV has a large DNA genome (151 kb) and large inserts of DNA can be incorporated into the LSDV genome (Tulman *et al.*, 2001). Genetically, LSDV closely resembles the other members of the *Chordopoxviradae*, although it contains a unique set of genes that correspond to its host range and virulence (Tulman *et al.*, 2001).
The attenuated vaccine strain of LSDV (Neethling) has been shown to be a safe and efficacious vaccine vector (Aspden *et al.*, 2002; Aspden *et al.*, 2003; Kitching *et al.*, 1987; Wallace *et al.*, 2006; Wallace & Viljoen, 2005). The safety of LSDV (Neethling) has been demonstrated through the vaccination of cattle against Lumpy skin disease (LSD) for many years without any complications (Kitching *et al.*, 1987). The restriction in the host-range of LSDV reduces the risk of possible dissemination of LSDV in non-ruminant animals or humans. LSDV does not transmit horizontally from vaccinated to unvaccinated animals (Carn & Kitching, 1995; Kitching & Mellor, 1986), which adds another desirable property to the safety profile of LSDV as a vaccine vector.

1.2.2.3.1 LSDV as a vaccine vector

Recombinant LSDV vaccines (rLSDV) have been constructed against several livestock

infectious diseases, namely, rinderpest virus, rabies virus and Ehrlichia ruminantium Welgevonden infections (Aspden *et al.*, 2002; Ngichabe *et al.*, 2002; Pretorius *et al.*, 2008; Romero *et al.*, 1993; Romero *et al.*, 1994b; Wallace *et al.*, 2006). Dual protection against LSD and the pathogen of the recombinant antigen expressed, can be provided. Vaccination of cattle with the recombinant LSDV, strain KS-1, expressing both the haemagglutinin and fusion proteins of rinderpest virus, provided protection against lethal challenges of both rinderpest virus and LSDV (Ngichabe *et al.*, 1997; Romero *et al.*, 1993; Romero *et al.*, 1994a). Long-term protective immunity induced by the recombinant LSDV against both viruses was also demonstrated (Ngichabe *et al.*, 2002). Two LSDV recombinants expressing Blue tongue virus and peste-des-petits-ruminants virus antigens respectively have also been tested. These vaccines induced partial protective immunities in goats and sheep against the respective pathogen challenges (Berhe *et al.*, 2003; Perrin *et al.*, 2007).

The efficacy of LSDV as a replication-deficient viral vector has also been demonstrated in non-ruminant hosts. A recombinant LSDV expressing the glycoprotein of rabies virus (Aspden *et al.*, 2002; Aspden *et al.*, 2003) was found to elicit rabies glycoprotein specific antibodies in both cattle (ruminant) (Aspden *et al.*, 2002) and rabbits (non-ruminant) (Aspden *et al.*, 2003). The same recombinant LSDV did not induce a rabies glycoprotein specific antibody response in mice; however, rabies-specific cellular immune responses were elicited in mice, rabbits and cattle. The recombinant LSDV provided strong protection against high dose rabies virus challenge, and complete protection against low-dose rabies virus challenge in mice (Aspden *et al.*, 2003). The protection provided by this recombinant LSDV was comparable to the protection induced by a WHO approved commercial rabies vaccine Verorab (inactivated rabies virus) in mice (Aspden *et al.*, 2003).

A recombinant LSDV expressing the Rift Valley fever virus (RVFV) glycoprotein induced neutralising antibodies against RVFV in mice and sheep and completely protected the vaccinated animals against Rift Valley fever virus challenge (Wallace *et al.*, 2006; Wallace & Viljoen, 2005). The recombinant LSDV induced superior protective immune responses in mice compared to the immune responses induced by purified Rift Valley fever virus glycoprotein or DNA vaccine expressing the same glycoprotein (Wallace *et al.*, 2006; Wallace & Viljoen, 2005).

The block in the life cycle of LSDV in a non-permissive cell line (CV-1) was shown to occur at a late stage of the virus cycle, by infecting the cells with LSDV, transfecting with a plasmid containing the lacZ gene under the control of the vaccinia virus p11 promoter and detecting lacZ expression (Aspden *et al.*, 2003). LacZ expression from the late promoter would only have

occurred once viral DNA replication had taken place. The production of immature virions post virus genome replication was also observed using electron microscopy (Aspden *et al.*, 2003). The duration and level of the foreign antigen expressed in recombinant LSDV could therefore be superior to that of recombinant fowlpox and canarypox viruses, due to a much earlier block in the avipoxvirus replication cycle in non-permissive hosts (Taylor *et al.*, 1995). We speculate that the immunogenicity induced by recombinant LSDV will be superior to that induced by recombinant fowlpox and canarypox viruses, but comparable to recombinant MVA, which also progresses to the stage of forming immature virions (Sutter & Moss, 1992).

1.3 Project motivation

With the urgent need for a more efficacious HIV-1 vaccine and the limitations of the current available vaccine vectors, it is important to evaluate novel vaccine vectors which may be suitable for development into HIV-1 vaccines. Recombinant LSDV has been shown to be immunogenic and efficacious in a number of animal infectious disease models (Aspden *et al.*, 2002; Ngichabe *et al.*, 2002; Pretorius *et al.*, 2008; Romero *et al.*, 1993; Romero *et al.*, 1994b; Wallace *et al.*, 2006). The aim of this study was to evaluate LSDV as a novel HIV-1 vaccine vector. An important objective was to evaluate the safety of LSDV (Neethling) in an immunocompromised setting. This was done by injecting LSDV into two groups of mice with different immunodeficiency phenotypes and monitoring the weight loss, as an indication of pathogenicity. The next objective was to construct a recombinant LSDV (rLSDV-Grttn) expressing HIV-1 subtype C polyprotein Grttn (Gag, RT, Tat and Nef) and to evaluate it for immunogenicity in BALB/C mice. rLSDV-Grttn was administered as a single vaccination and in prime-boost combination with DNA or MVA vaccines expressing Grttn, in order to determine the optimum vaccination regimen. HIV-1 specific IFN-γ and IL-2 cytokine secretion was measured, as were surface molecules CD107a/b (degranulation correlate) and CD44 (antigen experienced status correlate) on the splenocytes of vaccinated mice. The presence of HIV-1 specific antibodies in the sera of vaccinated mice was also determined.

Chapter 2 Materials and Methods

2.1 DNA Procedures Employed in the Construction of Transfer Vectors

2.2 Lumpy Skin Disease Virus (LSDV) Propagation

2.3 Transient Expression of HIV-1 Polyprotein Grttn and Reporter Gene GUS

2.4 Construction and Purification of Recombinant LSDV Expressing HIV-1

2.1 DNA Procedures Employed in the Construction of Transfer Vectors

The cloning strategy followed in the construction of the transfer vector pYS-05 is described in chapter 3, section 3.2, pages 64- 72 (Figures 3.4, 3.8 and 3.10).

2.1.1 Source of plasmids used in the project

pLW-51 (Wang *et al.*, 2004) was kindly provided by Linda Wyatt (NIH, USA). HIV-1 subtype C polyprotein gene Grttn (Gag, RT, Tat and Nef) (Burgers *et al.*, 2006) with upstream Kozak sequence cloned into pMOSBlue was provided by Dr J. van Harmelen (Medical Virology, UCT). The plasmid pGPT07/14 (Boyle & Coupar, 1988), containing the *E.coli* xanthine-guanine phosphoribosyl transferase gene (Gpt) with upstream VV P7.5 promoter cloned into the *Eco*RI restriction enzyme site, was obtained as a gift from Dr Boyle (CSIRO, Australia) to Prof. Keith Dumbell (former member of the department).

2.1.2 DNA preparation

Small scale plasmid DNA preparation was done using the High Pure Plasmid Isolation Kit (Roche, Germany). Large-scale plasmid DNA preparation was carried out using the NucleoBond® 500-Maxi Kit (Clontech Laboratories, USA). Endotoxin-free plasmid DNA was prepared using the EndoFree Plasmid Maxi Kit (Qiagen, USA). DNA extraction from agarose gels was done using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). PCR products were purified using the MiniElute PCR purification kit (Qiagen, Valencia, CA). All kit procedures were performed according to the manufacturers' instructions and recommendations.

2.1.3 Polymerase chain reaction (PCR)

Oligonucleotide primers (Table 2.1) were ordered from the Synthetic DNA Laboratory in the Department of Molecular and Cell Biology, University of Cape Town. For the polymerase chain reaction (PCR), annealing temperatures were chosen approximately 5^0C below the melting temperature of the primers and extension times were chosen according to the expected size of the fragment to be amplified and the rate of polymerization of the DNA polymerase. The following general PCR cycling conditions were employed using either *Pfu* polymerase (Promega, USA) or GoTaq™ DNA Polymerase (Promega, USA): 95º C for 2 minutes, 30 cycles of 95º C for 1 minute, 50-55º C for 30s and 73-74º C for 2-5 minutes, followed by a final elongation process at 73-74º C for 5-9 minutes.

Table 2.1 PCR primer pairs used for DNA amplification of 5' and 3' end of LSDV RR fragments (LFI and LFII respectively), HIV-1 Grttn (Grttn) and *E.coli* Gpt (Gpt).

Primer name	Amplified product	Sequences
LFI forward	LFI	5'-GAATTCATGGTATAAAATAAAATGGAACC-3'
LFI reverse	LFI	5'-GCGCGCCAAACGCTATTAATCGTTCTC-3'
LFII forward	LFII	5'-CTGCAGTTGAGGGAATATTCTTTTCCGG-3'
LFII reverse	LFII	5'-AAGCTTGGTTATTCAAGATAATTAACAAGAG-3'
Grttn forward	Grttn	5'- ACCATGGCTGCTCGCGCATC -3'
Grttn reverse	Grttn	5'- TCAGTCCTTGTAGTACTCGG -3'
Gpt forward	Gpt	5'- ATTGCTCTTTCGGTGGCTGG -3'
Gpt reverse	Gpt	5'- CAACCCTCAAGAACCTTTG -3'

2.1.4 Agarose gel electrophoresis

Electrophoresis of DNA was carried out through ethidium bromide (0.5 µg/ml) stained 1% agarose gels (13 cm) prepared in Tris-acetate-EDTA (TAE, 0.04M Tris, 128 mM EDTA, 0.11% Glacial acetic acid) buffer, under constant voltage (V) of 80 V for 1 hour. Gels were visualized on an ultraviolet (UV) transilluminator.

2.1.5 Cloning

Restriction enzyme digestion and DNA cloning was performed according to standard procedures (Sambrook *et al.,* 1989). Ligation of DNA fragments was done using the Rapid DNA ligation kit (Roche, Germany) as instructed by the manufacturer. Competent cells were prepared from *E.coli* DH5α, using the $CaCl_2$ treatment procedure described by (Dagert & Ehrlich, 1979). Competent cells were transformed with plasmid DNA by incubating ligated DNA with thawed competent cells (100µl) for 20 minutes on ice. The cells were then heat-shocked at 42˚C for 50 seconds before adding 1 ml of 2X yeast-tryptone medium (2X YT, 1.6% Tryptone, 1% Yeast extract, 86 mM NaCl, pH 7) to the cells and incubating at 37˚C for 1 hour. The transformed cells were then plated onto ampicillin (Sigma-Aldrich, USA) containing 2X YT agar plates (2 x YT with 15% agar and 100µg/ml ampicillin) and further incubated at 37˚C overnight.

2.1.6 DNA sequencing

DNA sequencing was outsourced to the DNA sequencing unit in the Department of Molecular and Cell Biology, University of Cape Town. The analysis of sequencing data was performed using DNAMAN (Lynnon Corporation, Canada) and Chromas (Technelysium Pty Ltd, Australia) software.

2.2 Lumpy Skin Disease Virus (LSDV) Propagation

2.2.1 Viruses and cells

The Neethling strain of Lumpy Skin Disease Virus (LSDV), a bovine vaccine, was provided by Onderstepoort Veterinary Institute. MVA was obtained from Therion Biologic. Recombinant MVA expressing HIV-1 polyprotein Grttn under the control of VV mH5 promoter, inserted into the delIII region of MVA, was constructed by Nicolette Johnston (former member of the department). Propagation of LSDV was done in foetal bovine testes cells (FBT) as described below (section 2.2.2). Madin-Darby bovine kidney epithelial (MDBK) cells were obtained from the American Type Culture Collection (ATCC, CL-22) and used for LSDV titration.

2.2.2 Preparation of primary FBT cells from foetal bovine testes (Wallace, 1994)

LSDV grows optimally in foetal bovine primary cells. Primary foetal bovine testes cells were isolated from foetal bovine testes obtained from the Maitland abattoir in Cape Town. The testes were sliced open to remove the capsules, blood vessels and fat (Figure 2.1). The soft testes tissue was removed and cut into smaller pieces. The tissue was washed with 25% trypsin (Gibco) (in PBS) to rinse off the excess blood and then incubated with 1% trypsin at room temperature for 30 minutes. The trypsinized mix was filtered through a steel mesh and collected in 50 ml tubes (kept at 4˚C). Fresh 1% trypsin was added to the remaining tissue and the above process was repeated for another two cycles. The trypsinized mixes were pooled and centrifuged at 1000 rpm for 10 minutes to pellet the cells. The supernatant fluid was discarded and the cell pellet was resuspended in DMEM with 10% fetal calf serum (FCS, Gibco, USA). The resuspended cells were placed into tissue culture flasks and incubated at 37˚C, 5% CO_2 until the cells grew to confluency.

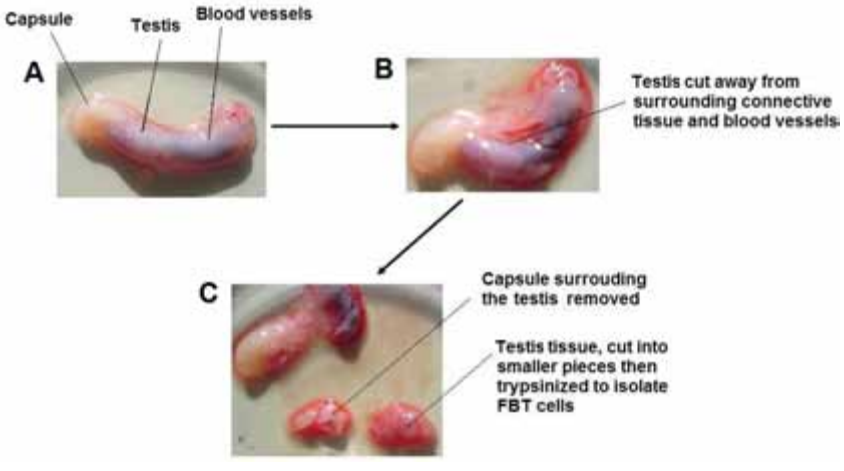

Figure 2.1 The isolation procedure of FBT cells from foetal bovine testes. A) Single detached foetal bovine testis, B) Detached foetal bovine testes with outer membrane removed. C) Soft testis tissue detached from the capsule and blood vessels.

2.2.2.1 Trypsinization and storage of FBT cells

80-90 % confluent FBT cells were trypsinized using activated trypsin solution (Trypsin/EDTA (Gibco, USA) diluted 1 in 10 with sterile PBS). The cells were washed with DMEM before centrifuging the cell suspension at 1000 rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 1/10th of the original growth medium volume of FCS + 10% DMSO. 1/2 and 1/10 dilutions of the cells were stained with Trypan Blue and counted using a Neubauer cell counting chamber. The concentration of FBT cells was adjusted to 3 x 10^6 cells/ ml with FCS + 10% DMSO, and then stored in aliquots at -80˚C.

2.2.2.2 Thawing FBT cells from frozen vials

Frozen FBT cells were thawed at 37˚C and resuspended in 10 ml of pre-warmed (37˚C) DMEM. The resuspended cells were centrifuged at 1000 rpm for 10 minutes, the cell pellet was resuspended in DMEM + 10% FCS, seeded into tissue culture flasks and incubated in a humidified incubator at 37˚C, 5% CO_2 for 48 hours. These FBT cells could be further passaged for 6-7 times before losing growth capability.

2.2.3. LSDV infection of primary FBT cells

2.2.3.1 Preparation of FBT cells

80-90 % confluent FBT cells were trypsinized as described in section 2.2.2.1, except the final cell pellet was resuspended in 1/10th of the original growth medium volume of DMEM + 10% FCS. The cells were counted and seeded at a concentration of 0.25 x 10^6 cells/ml. Volumes of 20 ml per tissue culture flask (175 cm^2) and 4 ml per well of a 6-well plate were used. The cells were incubated in a humidified incubator at 37˚C, 5% CO_2 for 24 hours.

2.2.3.2 Infection of cells

LSDV was diluted in DMEM. 80-90 % confluent FBT cells in tissue culture flasks were washed with PBS. Five milliliters of the diluted virus was adsorbed onto the cells for 1 hour at 37˚C, 5% CO_2. Fresh growth medium (DMEM + 4% FCS) was added to the wild-type LSDV infected FBT cells and selection medium (25 µg/ml mycophenolic acid, 250 µg/ml xanthine, 4% FCS in DMEM) was added to rLSDV-Grttn- infected FBT cells. The virus-infected FBT cells were incubated as before for a further 72 hours.

2.2.3.3 Large scale preparation of LSDV and rLSDV-Grttn

The titres of LSDV and rLSDV-Grttn were increased through repeated rounds of infection of fresh FBT cells with the respective viruses. Virus-infected cells were subjected to three cycles of

freezing and thawing. The cell suspensions were centrifuged at 2500 rpm for 5 minutes to pellet the cell debris. The virus-containing supernatants were pooled in a 175 cm^2 tissue culture flask and 45 ml aliquots were transferred to 50 ml oakridge centrifuge tubes (NALGENE Labware, Waltham, USA). 2 ml of 36% sucrose solution (in PBS) was slowly added to the bottom of each tube. The tubes were centrifuged at 11000 rpm for 1 hour at 4˚C to pellet the virus. The supernatant fluid was discarded. The pellet was washed gently with 30 ml of sterile PBS (Gibco, USA). The tubes were centrifuged as before for 30 minutes. The PBS was discarded and the virus pellets were resuspended in a total volume of 1 ml of sterile PBS. The resuspended virus was aliquotted into 50 µl aliquots in pyrogen-free Eppendorf tubes.

2.2.3.4 Titration of LSDV

The titre of LSDV was determined by counting foci of cytopathic effect (CPE) on the infected cells. Wild-type LSDV was titrated by counting foci using a light microscope and recombinant virus was titrated by immunostaining and counting brown foci, using a light microscope. The titration procedures are described below.

80-90% confluent MDBK cells were prepared in six well tissue culture plates as previously described for primary FBT cells (section 2.2.3.1). The virus to be titrated was diluted serially 10-fold (10^{-2} to 10^{-8}) in DMEM. The MDBK cells were washed twice with 1 ml of PBS before adding 300 µl of DMEM to each well. 200 µl of each virus dilution was vortexed and transferred to a designated well. The virus was allowed to adsorb for 1 hour at 37˚C, 5% CO_2. Medium was added to the wells of MDBK cells without removing the virus inoculae to make the final volume 2 ml in each well. Virus infected cells were incubated for 48 hours. For wt LSDV foci were counted directly using a light microscope. For recombinant LSDV the following staining procedure was followed.

The medium in the wells was aspirated, the cells were washed with 1 ml of PBS three times, PBS was completely aspirated from the wells and the cells were fixed with 1 ml of pre-cooled (-20ºC) methanol and acetone mix (1:1) for 2 minutes. The fixative was then removed and cells were washed three times with PBS as before. RT-specific sheep antibody was diluted 1 in 100 in PBS + 3% FCS and 750 µl was added to each well of MDBK cells. The cells were incubated for 1 hour with gentle agitation and washed with PBS as before. Horseradish peroxidase conjugated anti-sheep immunoglobulin (Dako, Denmark) was diluted in PBS + 3% FCS and 750 µl was added to each well. The cells were incubated for 45 minutes before washing with PBS as before. Substrate solution was prepared by adding 200 µl of o-Dianisidine (Sigma-Aldrich, USA) saturated ethanol and 10 µl of 30% hydrogen peroxide (Sigma-Aldrich, USA) to 10 ml of PBS.

The cells were incubated in substrate solution for 5-10 minutes. The colour development was stopped by washing the cells in distilled water. Orange foci were counted when viewed using a light microscope (400 X magnification).

The titre of rLSDV-Grttn was calculated by multiplying the counted RT positive foci by the dilution factor and multiplying by 5 to finally express the titre as foci forming units per millilitre (ffu/ml).

2.3 Transient Expression of HIV-1 Polyprotein Grttn and Reporter gene GUS

2.3.1 LSDV infection and pYS-05 transfection of FBT cells

Transient expression of the HIV-1 polyprotein Grttn and reporter gene GUS from the transfer vector pYS-05 occurs when the plasmid is present in the same FBT cell in which LSDV replication is taking place. pYS-05 was introduced into LSDV infected cells by transfection, as described below.

80-90 % confluent FBT cells were seeded into a six well tissue culture plate as described in section 2.2.3.1 The final volume seeded was 2 ml per well. Two wells of confluent FBT cells were infected with LSDV at a m.o.i of 0.1 ffu/cell. The volume of the virus inoculae used was 1 ml per well. Two wells were left uninfected as negative controls. 5 hours post infection, 100 ng of pYS-05 was transfected into one of the infected wells and one of the uninfected wells, using Effectene from an Effectene transfection kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The cells were incubated at 37˚C, 5% CO_2 for 24 hours before proceeding to the expression assays described below.

2.3.2 Detection of transient expression of reporter gene GUS from pYS-05 by substrate staining (GUS Staining)

After infection and transfection of FBT cells as described above (section 2.3.1), the cells were washed with 1 ml of DMEM per well, the medium was removed and 1 ml of staining overlay, consisting of DMEM/Hams F12 with 4 % FCS, 0.2 mg/ml X-Gluc (Clonetech, USA) and 1% low-melting point agarose, was added per well. The staining overlay was preheated at 45˚C before adding to each well. The staining overlay was incubated at room temperature until the agarose solidified. The cells were incubated at 37˚C, 5% CO_2 for 6 hours, before observing the blue coloration in the cell monolayer.

2.3.3 Immunofluorescence staining for transient expression of HIV-1 p24 (Gag) from pYS-05

Before seeding FBT cells into 6 well tissue culture plates, sterile round glass cover slips (1.2 cm in diameter) were placed into each well. The cells were seeded as described above (2.2.3.1). Confluent FBT cells on the cover slips were transferred to separate wells of a 24 well tissue culture plate. The cells were left uninfected, infected, transfected, or both infected and transfected. Infection was at a m.o.i. of 1 ffu/cell. One microgram of pYS-05 was transfected into designated FBT cells as described in section 2.3.1. The plates were incubated at 37°C, 5% CO_2 for 24 hours.

After incubation, the cells were washed with PBS, fixed with pre-cooled acetone methanol mix (1:1) at 4°C for 10 minutes, blocked with 5% bovine serum albumin (BSA) diluted with PBS (500 µl per well) for 30 minutes at room temperature, washed twice with PBS for 10 minutes per wash at room temperature and then incubated at 37°C for 1 hour with sheep anti-p24 antibody diluted 1/100 in 1.5% BSA (200 µl per well). The cells were then washed three times with PBS for 10 minutes per wash at room temperature, before being incubated with FITC conjugated donkey anti-sheep antibody (DAKO, Denmark) diluted 1/500 in 1.5% BSA. 200 µl of the antibody dilution was added to each cover slip. The adsorption was carried out at 37°C for 1 hour after which the cells were washed with PBS as before. The cover slips were then rinsed with distilled water, air dried, mounted on to glass slides with anti-fade mounting fluid (Sigma-Aldrich, USA) and viewed using a fluorescent microscope.

2.3.4 Detection of transient expression of HIV-1 RT from pYS-05 by Western blot analysis

2.3.4.1 Protein sample preparation

FBT cells, seeded into a six well tissue culture plate, were infected with LSDV and transfected with pYS-05 as described in section 2.3.1.

The FBT cells were washed with PBS and incubated with 200 µl of 1 x CAT lysis buffer (Roche, Germany), 1 x Complete® protease inhibitor (Roche, Germany) for 30 minutes at room temperature. The total protein concentration of each sample was determined using the Bio-Rad *DC* Protein Assay kit II according to the manufacturer's instructions. Sample absorbance readings were determined using a VERSAmax microplate reader.

Fifty micrograms of sample was used in a total of 40 µl including loading buffer (50 mM Tris-HCl; pH 6.8, 2% SDS, 10% Glycerol, 1% β-Mercaptoethanol, 12.5 mM EDTA and 0.02% Bromophenol Blue). The samples were heated at 100°C for 10 minutes and centrifuged briefly using a microfuge. 100 ng of RT protein and 10 µl of Precision Plus protein™ Kaleidoscope standard (Bio-Rad, USA) were also prepared for PAGE.

2.3.4.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A 10% separating gel (Bio-Rad, USA) was prepared. The mix was poured into assembled glass plates leaving 2 cm from the gel to the top of the plate. The gel was then topped up with 1 ml of isopropanol to ensure polymerization in the absence of oxygen. After 20 minutes the isopropanol was poured off and gel rinsed with distilled water. The remaining water droplet was removed using Whatman paper.

The 5% stacking gel solution was prepared (Bio-Rad, USA) and poured between the plates to the top. The comb was inserted into the unset stacking gel, which was left to polymerize for 45 minutes.

The plates were removed from the casting apparatus and clipped onto the electrophoresis apparatus. 1 x SDS buffer was added to the apparatus until the top of the small plate was covered. The comb was then removed from the gel and the wells were flushed with buffer to remove excess acrylamide. The marker and the samples were loaded into the wells and electrophoresis was carried out at 180 V for 45 minutes.

The gel was removed from the plates and soaked in transfer buffer (25 mM Tris and 192 mM glycine) for 30 minutes. Hybond™-P membrane (Amersham, USA) and blotting paper was cut to the same size as the gel. The pieces of blotting paper were soaked in transfer buffer until use. The membrane was soaked in methanol for 2 minutes, washed in water for 5 minutes, soaked in transfer buffer for 5 minutes and stored in transfer buffer until use. The transfer sandwich was set up on a BioRad SD semi-dry Electroblotting apparatus in the following order: blotting paper, gel, membrane and blotting paper. Air bubbles were removed before starting the electroblotting procedure. Electro blotting was done at 25 V for 1 hour. After disassembling the blotting sandwich the membrane was rinsed in 1 x TBS and stained with Ponceau S (0.1% Ponceau S, 5% acetic acid) for 2 minutes. The Ponceau S was then discarded and the membrane was rinsed with water until protein bands were visible. The membrane was then soaked in blocking buffer overnight at 4°C.

Sheep-anti RT antibody (1/2000 dilution) was prepared in a total of 20 ml blocking buffer. The blocking buffer was removed from the membrane. The prepared antibody was added to the membrane and left on a shaker to allow very gentle agitation for 2 hours. The blot was washed four times with blocking buffer for 15 minutes per wash with high speed shaking. The blocking buffer was then removed and anti sheep antibody conjugated with alkaline phosphatase, diluted 1 in 10000 (2 µl in 19.998 ml blocking buffer), was added to the membrane and left on a shaker at room temperature for 1 hour with gentle agitation.

The membrane was washed four times as before and rinsed in 1 x TBS. The membrane was kept in TBS until substrate solution was added. The substrate solution of alkaline phosphatase was prepared by dissolving 1 NIP/BCIP tablet (Roche, Germany) in 10 ml of water. The substrate solution was poured on to the membrane. The membrane was kept in the dark for 5-10 minutes for colour development.

2.4 Construction and Purification of Recombinant LSDV Expressing HIV-1 Polyprotein Grttn (rLSDV-Grttn)

2.4.1 rLSDV-Grttn construction

Recombinant LSDV was generated from homologous recombination between the transfer vector, pYS-05 and LSDV genomic DNA. pYS-05 was transfected into LSDV-infected FBT cells as described in section 2.3.1. Recombinant LSDV was isolated after repeated passage of the transfection lysate under Gpt selection conditions and detection of reporter gene, GUS, expression. The procedures performed are detailed below.

FBT cells were seeded into six well tissue culture plates as described in section 2.2.3.1. The cells were infected with LSDV diluted in 1ml DMEM, at a m.o.i of 0.1 ffu/ cell. Five hours post infection, FBT cells were transfected with 0.4 µg of pYS-05 as described in 2.3.1. Twenty-four hours post transfection, the FBT cells were washed with DMEM before adding selection medium (25 µg/ml mycophenolic acid, 250 µg/ml xanthine, 4% FCS in DMEM). The FBT cells were incubated as before for 72 hours. The virus was released from the FBT cells by freezing and thawing three times.

The harvested cell lysate was used to infect 80-90% confluent FBT cells seeded in a 25 cm^2 tissue culture flask. After one hour adsorption, the virus suspension was removed and selection medium was added. The FBT cells were incubated as before for 72 hours. The virus was

passaged in the same way four times before picking foci.

The virus suspension was diluted in serial 10 fold dilutions (10^{-2} to 10^{-7}). 80-90% confluent FBT cells seeded in six well tissue culture plates were infected with different dilutions of the virus. Seventy-two hours post infection GUS staining was performed on infected FBT cells as described in section 2.3.2. Six hours after adding the staining overlay, the plate was viewed using a light microscope at 100 x magnification. Blue foci were picked using p200 pipette tips and inoculated into 500 µl of DMEM. Virus from these foci was released with three cycles of freezing and thawing. Each picked focus was used to infected separate wells of 80-90% confluent FBT cells and incubated as before in selection medium. The process of infection and purification by focus picking was performed eight times until a pure recombinant LSDV (rLSDV-Grttn) stock was obtained.

The pick purified recombinant LSDV was propagated by infecting 80-90% confluent FBT cells in tissue culture flasks (Greiner, Germany) repeatedly. Virus infected cells were subjected to three cycles of freezing and thawing. The cell lysate was then centrifuged at 2500 rpm for 5 minutes to pellet the lysed cell debris. The virus-containing supernatant was then used to infect FBT cells in 10 flasks of 175 cm^2. Seventy-two hours post infection the virus-containing supernatant was purified as described in section 2.2.3.3 This propagation process of rLSDV-Grttn was repeated five times to increase the titre of rLSDV-Grttn.

2.4.2 Identification of wild-type and recombinant LSDV by PCR analysis

The presence of wild-type LSDV carried over from each pick-purification step was monitored by PCR. Three primers were designed: SQRR1, 5'-GTGGGCGTCAATGTTGAC-3', binds specifically to LSDV DNA sequences immediately upstream of the ribonucleotide reductase gene (insertion site). SQGrttn3, 5'-GCTACTTCCCCGACTGGC-3', binds specifically to sequences within and towards the end of the Grttn gene. Anti-sense primer SQRR2, 5'-CATAAAATCAGTACATGCATCC-3', binds specifically to LSDV sequences immediately downstream of the ribonucleotide reductase gene. The DNA fragments amplified from rLSDV-Grttn or wild-type LSDV can be distinguished by size differences; the DNA fragments amplified from rLSDV-Grttn and wild-type LSDV are 1.2 kb and 1.4 kb respectively. All three primers were added to each PCR reaction to detect both wild-type and recombinant virus in a single reaction.

Confluent monolayers of FBT cells in six well tissue culture plates were prepared as described in section 2.2.3.1. The cells were infected with rLSDV-Grttn or wild-type LSDV at an

approximate m.o.i. of 0.05 ffu/cell and DMEM with 4 % FCS (no selection) was added after viral adsorption. The plate was incubated at 37˚C, 5 % CO_2 for 48 hours. The cells were harvested by scraping into the growth medium and transferred to a 1.5 ml microfuge tube. The cell suspension was centrifuged at 14000 rpm for 10 minutes. The pellet was resuspended in 50 µl lysis buffer A (100 mM KCl, 10 mM Tris-HCl pH 8.3 and 2.5 mM $MgCl_2$). Fifty microlitres of lysis buffer B (10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$ and 1.5% Tween-20) was added to the suspension, followed by Proteinase K (Roche, Germany) (1 mg/ml). The suspension was incubated at 60˚C for 1 hour, followed by incubation at 90˚C for 10 minutes to inactivate the Proteinase K. This DNA extraction method was taken from (Kellogg & Kwok, 1990).

PCR was performed on the lysates using *Pfu* polymerase (Promega, USA) according to the manufacturer's guidelines. The PCR was carried out with an initial denaturing step at 95˚C for 2 minutes, followed by 35 cycles of 95˚C for 1 minute, 55˚C for 30 s, 73˚C for 5 minutes and a final elongation step of 73˚C for 7 minutes. The samples were then subjected to agarose gel electrophoresis (section 2.1.4).

2.4.3 Confirmation of HIV-1 polyprotein Grttn expression by rLSDV-Grttn using western blot analysis

The expression of full length Grttn by rLSDV-Grttn was confirmed using western blot analysis, using HIV RT- and Gag- specific antibodies. 80-90% confluent FBT cells in a six well tissue culture plate were 1) infected with rLSDV-Grttn (m.o.i of 0.01 ffu/cell), 2) infected with wild-type LSDV (m.o.i of 0.01ffu/cell) or 3) left uninfected.

Forty-eight hours post infection, protein samples of infected and uninfected FBT cells were prepared and subjected to SDS-PAGE and western blot analysis as described in section 2.3.4. A positive control of a lysate of SAAVI-MVA (recombinant MVA expressing HIV-1 subtype C Grttn and gp150 (Burgers *et al.*, 2008; Shephard *et al.*, 2008))-infected BHK-21 cells was available within the department.

2.5 Mouse Immunizations

2.5.1 Other vaccines used in the immunization

pVRCgrttnC, a DNA vaccine which codes for the HIV-1 polyprotein Grttn, was constructed by cloning Grttn with an enhancer intron A and a Kozak sequence, downstream of the cytomegalovirus AD169 immediate-early promoter (Burgers *et al.*, 2009; Shephard *et al.*, 2008).

rMVA-Grttn, a recombinant MVA expressing the same HIV-1 polyprotein Grttn, expressed from the same vaccinia virus H5 promoter as in rLSDV-Grttn, and also containing the reporter gene β-glucuronidase (GUS), was constructed by Nicolette Johnston (former member of the department). These genes were inserted into the deletion III region of the MVA genome.

2.5.2 Mice

All mouse procedures were done according to guidelines and approval of the University of Cape Town Animal Research Ethics Committee. RAG mice (lacking both T-cells and B-cells) and CD4 T cell knockout mice (6-8 weeks old) used for testing the safety of wild-type LSDV (wtLSDV), were purchased from Prof. Frank Brombacher (Immunology, IIDMM, UCT) and housed in a BSL-3 facility for the duration of the experiment. Female H-2^d BALB/C mice required for immunogenicity testing of rLSDV-grttn were purchased from South African Vaccine Producers (Pty) Ltd (Johannesburg, South Africa) and housed in a BSL-2 facility at the University of Cape Town Animal Unit. They were allowed to acclimatize for a minimum of 10 days prior to vaccinations.

2.5.3 Inoculation of immunocompromised mice with wild-type LSDV

Twenty RAG mice (4 groups) and 20 CD4 knockout mice (4 groups) were inoculated as indicated in Table 2.2. Two different doses of wtLSDV (10^4 and 10^6 ffu per mouse) were tested. Naïve mice and PBS vaccinated mice served as controls. The well-being of the mice (active and inquisitive when disturbed with clean and groomed hair coat) was observed daily and their weight recorded and plotted on a daily basis. On day 6, day 11 and day 30 post vaccination, differences in weight from that prior to vaccination were calculated as a percentage of pre-vaccination weight and presented as percentage weight change. This data for these days was statistically analysed for all mouse groups using the one-way ANOVA test with 95% confidence interval to determine the possible effect of wtLSDV on weight change.

Table 2.2. Safety of LSDV in immunocompromised mice. Inoculation and sacrifice schedule for groups of CD4 knockout mice and RAG mice (five mice per group).

Mouse Group with 5 mice per group	RAG/CD4 knockout	Day 0	Day1-Day29	Day 30
1	CD4 knockout	Weigh mice, intramuscular vaccination with PBS 100 µl	Monitor weight and appearance of mice	Monitor weight then sacrifice
2	CD4 knockout	Weigh mice, intramuscular vaccination with wtLSDV/10^4 ffu/100 µl	Monitor weight and appearance of mice	Monitor weight then sacrifice
3	CD4 knockout	Weigh mice, intramuscular vaccination withwtLSDV/10^6 ffu/100 µl	Monitor weight and appearance of mice	Monitor weight then sacrifice
4	CD4 knockout	Weigh mice	Monitor weight and appearance of mice	Monitor weight then sacrifice
5	RAG	Weigh mice, intramuscular vaccination with PBS 100 µl	Monitor weight and appearance of mice	Monitor weight then sacrifice
6	RAG	Weigh mice, intramuscular vaccination with wtLSDV/10^4 ffu/100 µl	Monitor weight and appearance of mice	Monitor weight then sacrifice
7	RAG	Weigh mice, intramuscular vaccination with wtLSDV/10^6 ffu/100 µl	Monitor weight and appearance of mice	Monitor weight then sacrifice
8	RAG	Weigh mice	Monitor weight and appearance of mice	Monitor weight then sacrifice

2.5.4 Immunogenicity of rLSDV-Grttn in combination with a DNA vaccine (pVRCgrttnC)

Forty-five 8-10 weeks old female BALB/C mice in 9 groups with 5 mice per group were vaccinated as indicated in Table 2.3. The mice were pre-bled one week before the first inoculation. Wild-type MVA (wtMVA) and wtLSDV served as control vaccines. The viruses (10^6 ffu in 100 µl PBS) and the DNA vaccine pVRCgrttn (100 µg DNA in 100 µl PBS) were injected by the intramuscular route, with 50 µl injected into each quadricep muscle. At the end of the experiment on day 40, blood was collected from the mice by cardiac puncture followed by cervical dislocation and harvest of the spleens (Table 2.3).

Table 2.3 Immunogenicity of rLSDV-Grttn alone and when used as a booster vaccine after a prime with pVRCgrttnC (DNA vaccine). Vaccination and sacrifice schedule for 9 groups of BALB/c mice (five mice per group). wtMVA and wtLSDV were used as controls.

Mouse Group with 5 mice per group	**Day -7**	**Day 0**	**Day 28**	**Day 40**
1	Pre-bleed	Intramuscular vaccination pVRCgrttnC 100 µg/100 µl	-	Bleed, sacrifice and harvest spleens
2	Pre-bleed	-	Intramuscular vaccination wtLSDV/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
3	Pre-bleed	-	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
4	Pre-bleed	-	Intramuscular vaccination rMVA-Grttn/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
5	Pre-bleed	Intramuscular vaccination pVRCgrttnC 100 µg/100 µl	Intramuscular vaccination wtLSDV/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
6	Pre-bleed	Intramuscular vaccination pVRCgrttnC 100 µg/100 µl	Intramuscular vaccination wtMVA 10^6 pfu/100 µl	Bleed, sacrifice and harvest spleens
7	Pre-bleed	Intramuscular vaccination pVRCgrttnC 100 µg/100 µl	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
8	Pre-bleed	Intramuscular vaccination pVRCgrttnC 100 µg/100 µl	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Bleed, sacrifice and harvest spleens
9	Pre-bleed	Intramuscular vaccination pVRCgrttnC 100 µg/100 µl	Intramuscular vaccination pVRCgrttnC 100µg/100 µl	Bleed, sacrifice and harvest spleens

2.5.5 Immunogenicity of rLSDV-Grttn in combination with rMVA-Grttn

2.5.5.1 Experiment A) Immunogenicity of the poxvirus prime/boost regimens

Forty-five 8-10 weeks old female BALB/C mice in 9 groups of 5 were vaccinated as indicated in Table 2.4A. The mice were pre-bled one week before the first inoculation. For these experiments wtMVA and wtLSDV served as control vaccines. The viruses (10^6 ffu in 100 µl PBS) were injected by the intramuscular route, with 50 µl injected into each quadricep muscle. At the end of the experiment on day 40, blood was collected from the mice by cardiac puncture followed by cervical dislocation and harvest of the spleens (Table 2.4A).

2.5.5.2 Experiment B) Characterization of the immunogenicity of poxvirus prime/boost regimens

Fifteen 8-10 weeks old female BALB/C mice in 3 groups of 5 were vaccinated as indicated in Table 2.4B. The mice were pre-bled one week before the first inoculation. wtMVA and wtLSDV served as control vaccines. The viruses (10^6 ffu in 100 µl PBS) were injected by the intramuscular route, with 50 µl injected into each quadricep muscle. At the end of the experiment on day 40, blood was collected from the mice by cardiac puncture, followed by cervical dislocation and harvest of the spleens (Table 2.4B).

Table 2.4A Immunogenicity of rLSDV-Grttn alone and in homologous and heterologous and prime-boost combinations with rMVA-Grttn. Vaccination and sacrifice schedule for 9 groups of BALB/c mice (five mice per group). wtMVA and wtLSDV were used as controls.

Mouse Group with 5 mice per group	**Day -7**	**Day 0**	**Day 28**	**Day 40**
1	Pre-bleed	-	Intramuscular vaccination wtLSDV/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
2	Pre-bleed	-	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
3	Pre-bleed	-	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Bleed, sacrifice and harvest spleens
4	Pre-bleed	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Intramuscular vaccination wtMVA 10^6 pfu/100 µl	Bleed, sacrifice and harvest spleens
5	Pre-bleed	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Intramuscular vaccination wtLSDV/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
6	Pre-bleed	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens
7	Pre-bleed	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Bleed, sacrifice and harvest spleens
8	Pre-bleed	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Bleed, sacrifice and harvest spleens
9	Pre-bleed	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Bleed, sacrifice and harvest spleens

Table 2.4B Characterization of the immunogenicity of heterologous poxvirus prime/boost regimens. Vaccination and sacrifice schedule for 3 groups of BALB/c mice (five mice per group) in heterologous prime/ boost vaccination regimens deploying rLSDV-Grttn and rMVA-Grttn.

Mouse Group With 5 mice per group	Day -7	Day 0	Day 28	Day 40
1	Pre-bleed	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Sacrifice and harvest spleens
2	Pre-bleed	Intramuscular vaccination rMVA-Grttn/10^6 pfu/100 µl	Intramuscular vaccination rLSDV-Grttn/10^6 ffu/100 µl	Sacrifice and harvest spleens
3	Pre-bleed	Intramuscular vaccination wtMVA 10^6 pfu/100 µl -	Intramuscular vaccination wtLSDV/10^6 ffu/100 µl	Sacrifice and harvest spleens

2.6 Immunogenicity Assays

2.6.1 Isolation of mouse splenocytes

After the mice were sacrificed, spleens were harvested and pooled from 5 mice per group into ice cold RPMI.

The pool of spleens from a group of mice was poured on to a cell strainer (pore size= 70µm) with a sterile petri dish below. The spleens were meshed gently through the cell strainer with a rubber plunger of a 2 ml syringe. The remaining cells were washed off the strainer with RPMI into the collecting petri dish. The meshed spleens were transferred to sterile 50 ml conical tubes. Collecting petri dishes were washed with RPMI and the solutions were transferred to the same tube. The volume of the cell suspension in each tube was adjusted to 50 ml using RPMI. The cells were pelleted by centrifuging the tubes at 400 g for 5 minutes. The supernatant was decanted and the cell pellet was gently resuspended in 50 ml of RPMI. The cells were then washed twice more by repeating these steps. After the last resuspension step, fibrin clots were removed with a sterile Pasteur pipette. Cells were resuspended in 50 ml RPMI and cell number and viability was determined by counting an aliquot of the cells in a Neubauer counting chamber after staining them with trypan blue stain. Red blood cells (RBCs) were lysed by suspending $50x10^6$ cells in 1ml of RBC lysis buffer (5 mM Tris-HCL, 140 mM NH_4Cl, pH 7.3, Sigma-Aldrich, USA) for 2 minutes. The cells were then pelleted by centrifugation at 400 g for 7

minutes. The supernatant containing the lysed red cells was discarded and splenocytes were resuspended in 5 ml RPMI and an aliquot of this suspension was stained with trypan blue and counted in a Neubauer chamber. Cells were then suspended at a final concentration of 5 x 10^6 cells/ml in R10 medium (RPMI supplemented with 1% penicillin G/streptomycin, 10% FCS and 0.1% 2-mercaptoethanol). For the experiment detailed in Table 2.4B where the effect of FCS on background responses in the IFN-γ ELISPOT was investigated the cells were resuspended at 5 x 10^6 cells/ml serum free medium (SFM; CTL TEST™ medium from Cellular Technology Ltd, Cleveland, Ohio) supplemented with 200 mM of L-glutamine (Sigma-Aldrich, USA)

2.6.2 ELISPOT Assays

The IFN-γ and IL-2 ELISPOT kits from BD Bioscience were used for the ELISPOT assays. Instructions provided in the kit protocol were followed during the assay. The responses to each of the stimulant peptides or controls were tested in triplicate for splenocytes from each group of inoculated mice.

Twelve hours before the sacrifice of the inoculated mice, ELISPOT plate membranes were pre-wetted with 15 µl of 35% ethanol for one minute. The membranes were then washed three times with 200 µl of PBS. The PBS was discarded from the wells and the membranes were coated overnight at 4 ºC with 100 µl of IFN-γ or IL-2 coating antibodies (5 µg/ml diluted in PBS) per well.

The coating antibodies were then discarded and the membranes were washed once with R10 or CTL TEST™ medium and then blocked with 200µl of complete medium or CTL TEST™ medium for two hours at room temperature.

Splenocytes were stimulated with peptides corresponding to CD4+ and CD8+ T cell epitopes in Gag and RT. An irrelevant peptide was included as a negative control (Table 2.5). Peptides were used at 2 µg/ml in R10 or glutamine supplemented CTL TEST™ medium. The polyclonal stimulus ConA (5 µg/ml) was included as a positive control (no data for this response is included in the Result section).

Table 2.5 Control and peptide stimulants used in ELISPOT assays

Stimulant/ Control	Description	Peptide sequences
Con A	Assay positive control	N/A
irrelevant peptide	H-2K^d binding peptide (negative peptide control)	TYSTVASSL
GagCD8	Gag H-2^d –restricted class I peptide (CD8 peptide)	AMQMLKDTI
GagCD4(13)	Gag MHC class II-restricted peptide (CD4 peptide)	NPPIPVGDIYKRWIILGLNK
GagCD4(17)	Gag MHC class II-restricted peptide (CD4 peptide)	FRDYVDRFFKTLRAEQATQE
RT CD8	RT H-2^d –restricted class I peptide (CD8 peptide)	VYYDPSKDLIA
RT CD4	RT MHC class II-restricted peptide (CD4 peptide)	PKVKQWPLTEVKIKALTAI

After blocking, the R10 medium or CTL TEST^{TM} medium in the wells was discarded. Stimulant and control peptides and ConA were added to the allocated wells in triplicate for each group (100 µl/well). 100 µl of R10 medium or CTL TEST^{TM} medium were also included in triplicate as a background control for the assay. This was followed by adding 100 µl of the splenocyte suspensions into allocated wells on the plates. The plates were protected from light exposure by covering them with foil, which also prevented evaporation. The plates were then incubated at 37^{o}C, 5% CO_2, humidified air incubator for 23 hours.

After the incubation, the cell suspensions were discarded from the plates and the membranes were washed three times with deionised water. The water was removed from the wells, and the membranes were washed three times with PBS+ 0.05% Tween 20 (Sigma-Aldrich, USA). The PBS + 0.05% Tween 20 was then discarded and a volume of 100 µl of biotinylated anti- IFN-γ or IL-2 detection antibody (2µg/ml in PBS with 10% FCS) was added to each well. The plates were incubated at room temperature in the dark for two hours after which the antibodies were discarded and the membranes washed three times with PBS+ 0.05% Tween 20. A volume of 100µl of avidin-horseradish peroxidase (Avidin-HRP) conjµgated anti- IFN-γ or IL-2 detection antibodies (5 ug/ml in PBS with 10% FCS) was added to each well and the plates were incubated at room temperature in the dark for one hour. The antibody was discarded and the membranes were washed three times with PBS+ 0.05% Tween 20 then three times again with PBS (Sigma-Aldrich, USA). The substrate solution Nova Red (Vector) was prepared in distilled

water and a volume of 100 µl was added to each well. The plates were incubated at room temperature in the dark for 5 minutes for spot development. The reaction was stopped by washing five times with distilled water. The plates were dried in the dark before analysis.

The ELISPOT plates were scanned using an ImmunoSpot CTL Reader and the spots in each well were counted using ImmunoSpot Software version 3.2. The software parameters indicated in Table 2.6 were used to avoid the counting of spots due to debris or mechanical damage.

Table 2.6 ImmunoSpot Software parameters used for ELISPOT data acquisition

Parameters name	**Parameter status**
Sensitivity	140
Minimum spot size	0.0025 mm^2
Maximum spot size	0.2907 mm^2
Oversized spots	Estimated
Spot separation	1.00
Diffuse spot process	On
Diffuseness	40
Gradient	Off
Overdeveloped area handling	Active
Auto areas	Estimated
Manual Areas	Normalized
Colour system	Red
Detailed counting	Off
Background balance	On at 80
Fill holes	Off
Audit spots	Inactive

For each reaction, the mean number of spots from triplicate wells and the standard deviation (SD) of this mean were calculated and adjusted to 1 x 10^6 splenocytes and expressed as spots forming units per 1 x 10^6 splenocytes (SFU/10^6 splenocytes).The mean number of spots and its SD in the absence of peptide for each group was used as the background response for the group. Positive responses of the splenocytes to HIV-1 peptides in each group were considered to be those greater than the background response plus two times the SD of this background response. For all positive responses, the background responses of the group were subtracted from the HIV-1 specific responses and the response then expressed as net SFU/10^6 splenocytes.

2.6.3 Quantification of antigen-specific cytokine production

Aliquots of the splenocytes pooled from 5 mice per group were also used to quantify the cytokines released from the splenocytes during culture with Gag and RT peptides. Splenocytes were adjusted to 15 x 10^6 splenocytes/ml with R10 medium and 100 µl of the splenocyte suspension (1.5 x 10^6 splenocytes) was added to each well in a 96 well round-bottom micro-titer plate followed by the addition of 100 µl of R10 medium, ConA or stimulant peptides (as described in Table 2.5 and used in the ELISPOT assays). The plate was incubated at 37°C, 5% CO_2 in a humidified air incubator for 48 hours after which 150 µl of the supernatant from each well was harvested without disturbing the cell pellets. The supernatants were then stored at -20°C until the cytokine content was determined using a BD™ cytometric bead array (CBA) mouse Th1/Th2 cytokine kit (BD Biosciences, USA) that measures IFN-γ, TNF-α, IL-2, IL-5 and IL-4.

The CBA assay was carried out according to the kit protocol. Briefly, lyophilized mouse Th1/Th2 standards were transferred to a FACS tube and reconstituted in 2 ml of assay diluent. The reconstituted standards were allowed to equilibrate at room temperature for 15 minutes. Serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256) of the reconstituted cytokine standards were made in FACS tubes. Three hundred microlitres of assay diluent were first pipetted into each of these tubes. A tube with assay diluent only was also prepared as a negative control.

The number of assays including the standards and the controls were determined. Individual capture beads (A1-A5, IL-2, IL-4, IL-5, IFN-γ and TNF-α capture beads respectively) were vortexed vigorously before pooling specific volumes of capture beads together in a FACS tube (10 µl of each type of capture bead per assay). The bead mixture was then mixed thoroµghly again.

The assays were set up in FACS tubes. 50 µl of capture bead mixtures were added to each tube. 50 µl of each standard dilution were added into allocated tubes. 50 µl of test sample supernatants were added into allocated tubes.

Fifty microlitres of Th1/Th2 PE detection reagents were then added into each of the tubes containing standards and test samples. The tubes were incubated at room temperature for two hours in the dark.

After two hours incubation, 1 ml of wash buffer was added to each of the tubes. The tubes were

then centrifuged at 200 g for 5 minutes. The supernatants were carefully discarded and each bead pellet was resuspended with 300 µl of wash buffer. The samples were then analyzed by flow cytometry as recommend by the instructions in the kit.

2.6.4 Staining of splenocytes with MHC-1 H-2D^k pentameric peptide complexes

2.6.4.1 Staining strategy for HIV-1 specific, CD8 + and CD44+ T cells

HIV-1 Gag- or RT- specific CD8+ T cells in the spleens were detected by binding of pentameric H-2D^k complexes folded with the Gag CD8+ or RT CD8+ peptide (see Table 2.5) conjugated to APC (Proimmune, Oxford, UK) and binding of anti-CD8 antibody conjugated with PerCP (Pharmingen). The antigen-experienced marker CD44 on these T cells was detected using an anti-CD44 antibody conjugated to FITC (Pharmingen). Non-specific binding of the pentameric complex to B-cells and monocytes was eliminated by gating on a CD45R/B220 and CD14 negative cell populations defined using CD45R/B220 and CD14 antibodies conjugated with PE (Pharmingen).

3 x 10^6 splenocytes, isolated from groups of mice vaccinated according to Table 2.4B, resuspended in CTL TEST^{TM} medium, were aliquoted per FACS tube. 2 tubes were prepared for each group (one tube for Gag pentameric complex binding, one tube for RT pentameric complex staining). The aliquoted cells were centrifuged at 500 g for 5 minutes at 4ºC and the CTL TEST^{TM} medium was discarded. The cells were resuspended in residual volume in the tubes for the staining process. A volume of 30 µl of Gag or RT pentameric complexes was added to the designated tubes and mixed. The cells were then incubated for 10 minutes at room temperature, shielded from light. During the incubation, an antibody mix solution containing anti-CD8-perCP, anti-CD45R/B220-PE, anti-CD44-FITC and CD14-PE was prepared in FACS buffer (0.1% Sodium Azide, 1% foetal calf serum in PBS) according to Table 2.7.

Table 2.7. Antibodies used in addition to the APC-conjugated Gag or RT pentameric complexes to select for Gag or RT specific CD44+, CD8+ T cells.

Antibody-conjugated fluorochrome	Working concentration of each antibody in the antibody mix (mg/ml)
CD8-PerCP	0.04
CD45R/B220-PE	0.002
CD44-FITC	0.00125
CD14-PE	0.004

After 10 minutes incubation, 150 µl of the antibody mix solution was added to the cells. The cells were incubated at 4ºC for 30 minutes, shielded from light, then washed with 1 ml of FACS buffer, followed by an addition of 1 ml FACS lyse solution, then incubated at room temperature for 10 minutes in the dark. The cells were then centrifuged as before. After discarding the supernatant, the cells were washed twice with 1 ml FACS buffer. The cells were then resuspended in 900 µl of FACS buffer per tube and analyzed by flow cytometry. The gating strategies used in flow cytometric analysis for Gag- or RT- specific/ CD44+/ CD8+ T cell populations are illustrated in Figure 2.2.

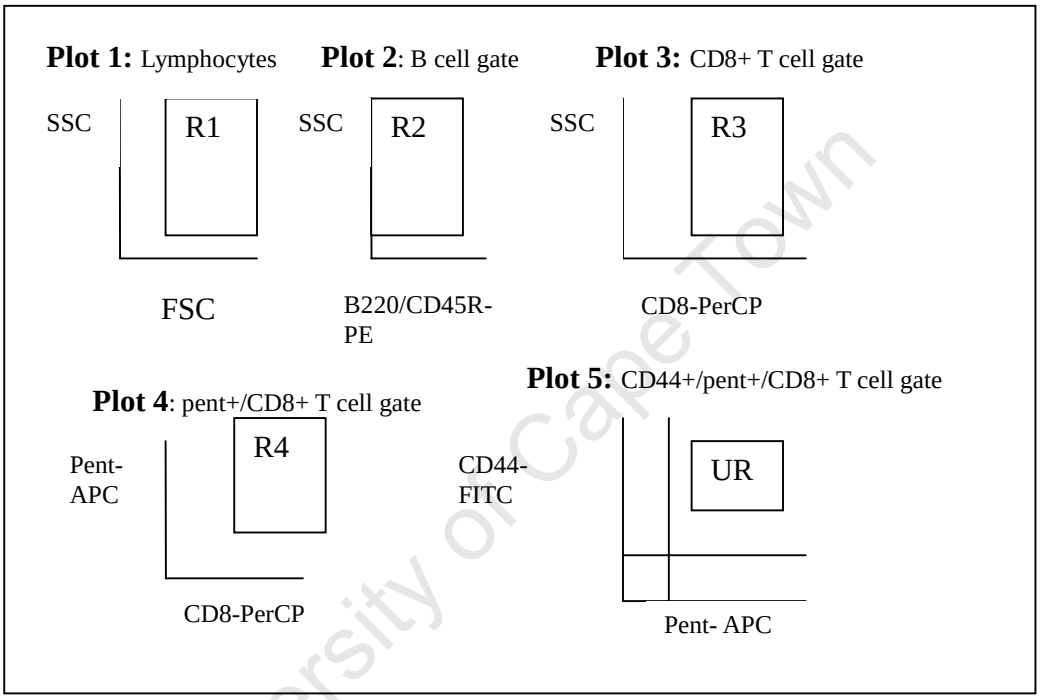

Figure 2.2 Cell gating strategies used in flow cytometric analysis for Gag- or RT- specific/ CD44+/ CD8+ T cells. Progressive gating: Lymphocytes (plot 1, R1), B-cells (plot 2, R2), CD8+ T cells (plot 3, R3), Pentameric H-2D^k complexes folded with the Gag CD8+ or RT CD8+ peptide (pent)+/CD8+ T cells and pent+/CD44+/ CD8+ T cells (Plot 5, UR).

2.6.4.2 Staining strategy for HIV-1 specific, CD8 + and CD107a/b+ T cells

Splenocytes (from groups of mice vaccinated as indicated in Table 2.4) in CTL TEST^{TM} medium were aliquoted at 3 x10^6 splenocytes per FACS tube. Four tubes were prepared for each group (two tubes for Gag pentameric complex and RT pentameric complex staining with peptide stimulations, two tubes for Gag pentameric and RT pentameric staining without peptide stimulations).

3 tests of Gag or RT pentameric complexes (30 µl) were added to the designated tubes with the splenocytes and mixed by pipetting. The cells were then incubated for 10 minutes at room

temperature, shielded from light. The splenocytes were washed with 2 ml of CTL TEST™ medium, and then centrifuged at 500 g for 5 minutes at 4ºC. The CTL TEST™ medium was then discarded and the splenocytes were resuspended in 300µl of CTL TEST™ medium per tube.

Antibody mix solution described in Table 2.8 were prepared for stimulated splenocytes and unstimulated splenocytes.

Table 2.8 Antibody mix and their volume required during peptide stimulation**.**

		Stimulated	**Unstimulated**
Antibody-conjugated fluorochrome	**Stock concentration (mg/ml)**	**Volume (µl)**	**Volume (µl)**
CD107a-FITC	0.5	6.0	6.0
CD107b- FITC	0.5	3.0	3.0
Anti-CD28	1.0	1.2	1.2
Anti-CD49d	1.0	1.2	1.2

After a 10 minute incubation, the splenocytes were washed as before then resuspended in 300 µl of CTL TEST™ medium per tube. The antibody described in Table 2.8 was added to the Gag or RT pentameric complex stained splenocytes in designated tubes. In the stimulated splenocytes, 0.6 µl of Gag CD8 or RT CD8 peptide (4 µg/ml, Table 2.5) was added to the splenocytes in the appropriate tubes. In the unstimulated splenocytes, 0.6 µl of CTL TEST™ medium was added to splenocytes in each of the appropriate tubes. The volume in each tube was adjusted to 600µl with CTL TEST™ medium and the tubes were incubated at 37˚C, 5% CO_2 in a humidified air incubator for 1.5 hours. BD GolgiStop™ containing monensin (1.2 µl, BD Bioscience, USAs) was added to each tube and the incubation continued for a further 4.5 hours.

After the incubation the splenocytes were washed with 1 ml CTL TEST™ medium. Antibody mix solution containing anti-CD8-perCP, anti-CD45R/B220-PE, and CD14-PE were prepared in FACS buffer according to Table 2.9.

Table 2.9 Antibodies used in addition to the Gag or RT pentameric complexs and CD107a/b antibody in selecting Gag or RT specific, CD107a/b+, CD8+ T cells

Antibody-conjugated fluorochrome	Working concentration of each antibody in the antibody mix (mg/ml)
CD8-PerCP	0.04
CD45R/B220-PE	0.002
CD14-PE	0.004

Volumes of 150 µl of the antibody mix solution described in Table 2.9 were added into each tube of cells. The tubes were incubated at 4ºC for 30 minutes, shielded from light. The cells were then washed with 1 ml of FACS buffer per tube, followed by an addition of 1 ml FACS lyse solution. The cells were further incubated at room temperature for 10 minutes, shielded from light. The cells were then centrifuged as before. After discarding the supernatant, the cells were then washed with 1 ml FACS buffer per tube and centrifuged as before twice. The cells were then resuspended in 900 µl of FACS buffer per tube and analysed by flow cytometry. The gating strategies used in flow cytometric analysis for Gag- or RT- specific/ CD107a/b+/ CD8+ T cell populations are illustrated in Figure 2.3.

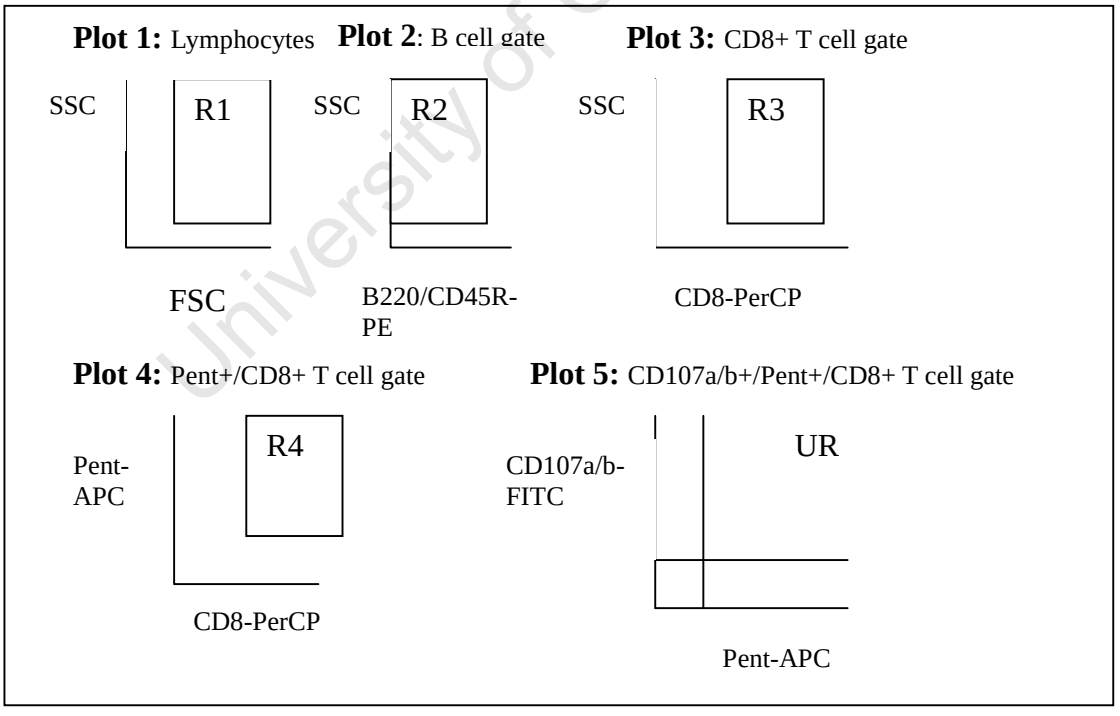

Figure 2.3 Cell gating strategies used in flow cytometric analysis for Gag- or RT- specific/ CD107a/b+/ CD8+ T cells. Progressive gating strategy: Lymphocytes (plot 1, R1), B-cells (plot 2, R2), CD8+ T cells (plot 3, R3), Pentameric H-2D^k complexes folded with the Gag CD8+ or RT CD8+ peptide (pent)+/CD8+ T cells and pent+/CD107a/b+/ CD8+ T cells (Plot 5, UR).

2.6.5 New LAV Blot assay

The New LAV blot Assay kit (Bio-Rad), a western blot based human HIV-1 specific antibody detection kit, was used according to the manufacturer's instructions. Briefly, the serum from the vaccinated groups of mice described in Table 2.3 and 2.4A, obtained during pre-bleed and post sacrifice were pooled separately within each group. The membrane strips were placed into each well with the labeling number and reference mark facing upwards. Two millilitres of the diluted wash buffer was added to each strip and incubated at room temperature for 5 minutes with gentle shaking.

Twenty microlitres of the positive and negative control antibodies and 50 µl of each group of pooled serum was added to the corresponding strips into the 2 ml buffer that was already in the well. The strips were then incubated at room temperature for 2 hours with gentle shaking. After the incubation, the buffer in the wells was aspirated. Two millilitres of wash buffer was added to each well and removed immediately. Two millilitres of wash buffer was then added to each well and incubated for 5 minutes at room temperature with shaking. The process was repeated for another wash.

Because the kit was designed to detect human antibodies against HIV proteins, secondary antibody specific for mouse immunoglobulin was used for the serum samples from mice. Two millilitres of washing buffer was added to each of the strips 1-5. Four microlitres of alkaline phosphatase conjugated goat anti mouse IGG (Dako) was added to the buffer in which the strips probed with mice sera were. Two millilitres of the conjugate solution provided in the kit were added to the well containing the controls. The strips were incubated at room temperature for 1 hour with gentle shaking. After incubation the strips were washed as before with wash/diluent buffer three times.

Finally, the wash buffer was removed and 2 ml of development reagent was added to each strip and incubated at room temperature for 5 minutes. The reaction was then stopped by washing the strips with 2 ml of distilled water three times.

Chapter 3 Construction of Recombinant LSDV (rLSDV-Grttn) Expressing HIV-1 Subtype C Polyprotein Grttn

3.1 Introduction

3.1.1 Construction of recombinant poxviruses

Technologies of recombinant poxvirus production have enabled the use of genetically modified poxviruses for the molecular biology of the poxvirus, and also in recombinant poxvirus vaccine vector research (Souza *et al.*, 2005). Because the poxvirus genome is not transcribed by the host cellular enzymes, the poxvirus DNA is not infectious. Therefore, direct modification of extracted poxvirus DNA will not produce any infectious virion (Moss, 1996)

Recombinant poxviruses are generally constructed by insertion of foreign genes using a transfer vector containing the genes of interest flanked by poxvirus sequences. The latter allow for homologous recombination into a specific site of the poxvirus genome. In order to produce a recombinant poxvirus expressing foreign genes, it is essential to consider the unique properties of the poxvirus transcription system when designing the transfer vectors. Poxvirus transcription occurs in the cytoplasm of the infected cell and poxviral RNA polymerase is required for transcription of viral genes. Promoters are also specific for poxviruses. It is therefore essential to have a poxvirus promoter sequence upstream of the inserted foreign gene (Schnierle *et al.*, 2007). The foreign gene should also have its own translation initiation codon (ATG) and stop codon. There should be no poxvirus early transcription termination sequence (TTTTTNT) (Yuen & Moss, 1987) within the inserted genes to ensure the correct and complete expression of the genes.

The conventional approach to constructing a recombinant poxvirus is to introduce the foreign genes into a non-essential region of the poxvirus genome by homologous DNA recombination (Moss, 1996). This approach involves constructing an expression cassette on a transfer plasmid (Figure 3.1). The expression cassette would consist of several foreign genes under the control of upstream poxvirus promoters. These genes would include the antigen gene of interest if constructing a recombinant poxvirus vaccine, and possibly a reporter gene and a positive selection gene. The expression cassette has to be flanked by DNA sequences that are homologous to the two ends of the poxvirus non-essential gene or non-coding region in which the foreign DNA is to be inserted. The thymidine kinase (TK) gene has been used as a common non-essential target gene for foreign gene insertion into the poxvirus genome (Boyle & Coupar, 1988; Mackett *et al.*, 1982; Wallace & Viljoen, 2005). The inactivation of TK by foreign gene insertion allows the recombinant poxvirus to be selected in TK deficient cell lines (Mackett *et al.*, 1982), in the presence of a toxic thymidine analogue, 5-bromodeoxyuridine, lethal to TK

active wild-type poxvirus. Other insertion sites have also been demonstrated to be non-disruptive for the replication and growth of certain poxviruses, such as the Del III region of MVA (Wang *et al.*, 2004) and the ribonucleotide reductase (RR) gene of LSDV (Aspden *et al.*, 2002; Aspden *et al.*, 2003). In this study, we have chosen the ribonucleotide reductase gene as the insertion site for constructing a recombinant LSDV. The use of this gene as a site into which to insert foreign DNA into the LSDV genome has previously been demonstrated to be successful in our department (Aspden *et al.*, 2002). Additionally, an antibiotic resistance gene and reporter gene may be included in the expression cassette for the selection of recombinant poxviruses.

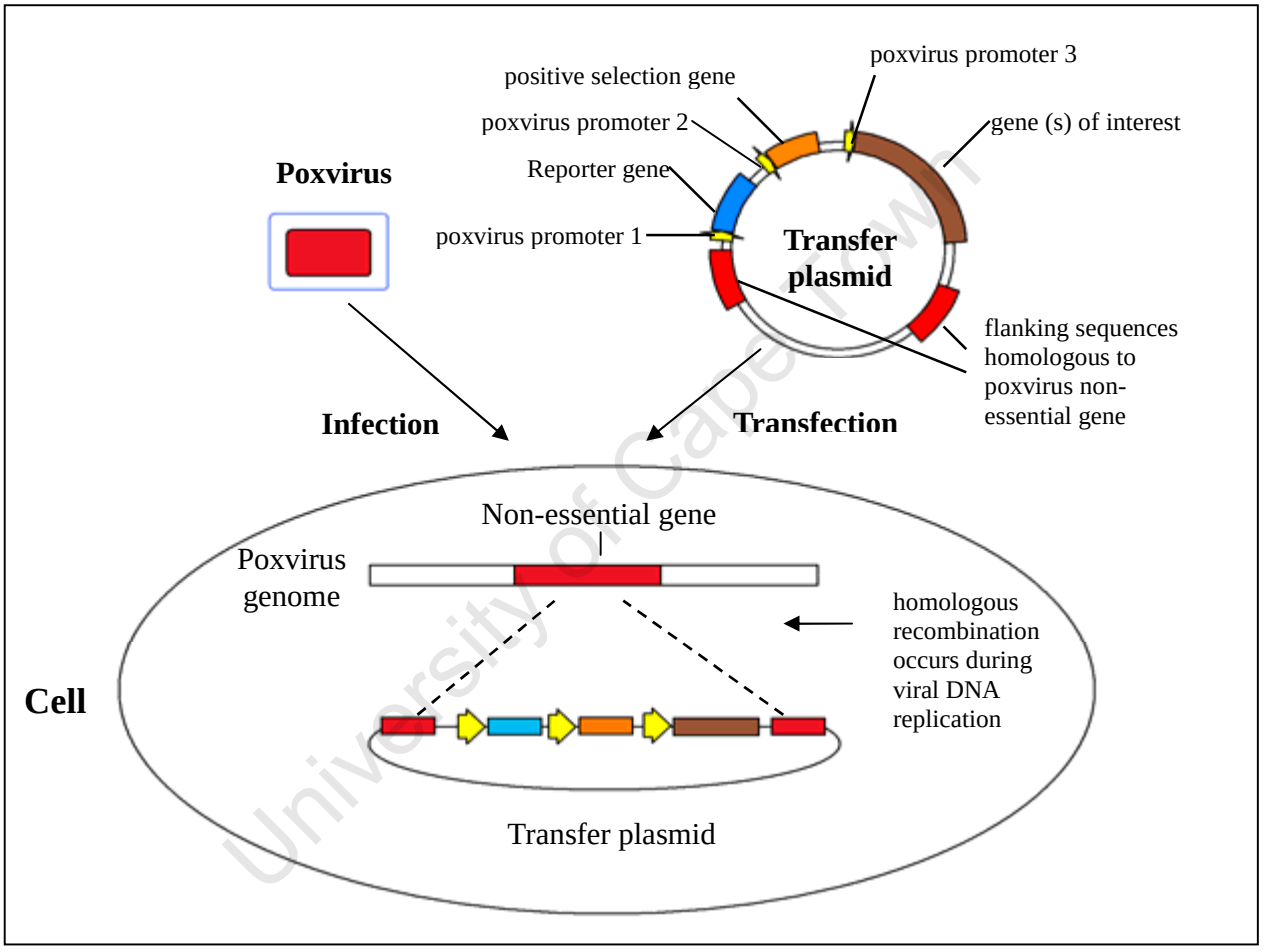

Figure 3.1 Production of recombinant poxvirus through DNA recombination between the viral genome and the transfer plasmid. The transfer plasmid has an expression cassette which consists of a reporter gene, a positive selection gene and the gene (s) of interest to be inserted into the poxvirus, all with suitable upstream poxvirus promoters. These genes are flanked by DNA homologous to the insertion site in the poxvirus genome, usually a non-essential gene. The transfer plasmid is transfected into poxvirus infected cells and homologous recombination takes place between the poxvirus non-essential gene and flanking DNA in the transfer plasmid during poxvirus DNA replication. The expression cassette in the transfer plasmid is inserted into the non-essential gene of the poxvirus genome. The recombinant virus can be detected and selected for by means of reporter gene and positive selection gene expression respectively. This figure is adapted and modified from (Schnierle *et al.*, 2007).

The *E.coli* xanthine-guanine phosphoribosyltransferase (Gpt) gene has been demonstrated to be a useful positive selection gene to be incorporated into the recombinant poxvirus (Boyle & Coupar, 1988). Mycophenolic acid (MPA) is an inhibitor of inosine monophosphate (IMP)

dehydrogenase, an enzyme involved in guanine nucleoside synthesis. In the presence of MPA and xanthine, Gpt can overcome the block in guanine nucleoside synthesis by utilizing xanthine as an alternative substrate for synthesis of GMP (Figure 3.2).

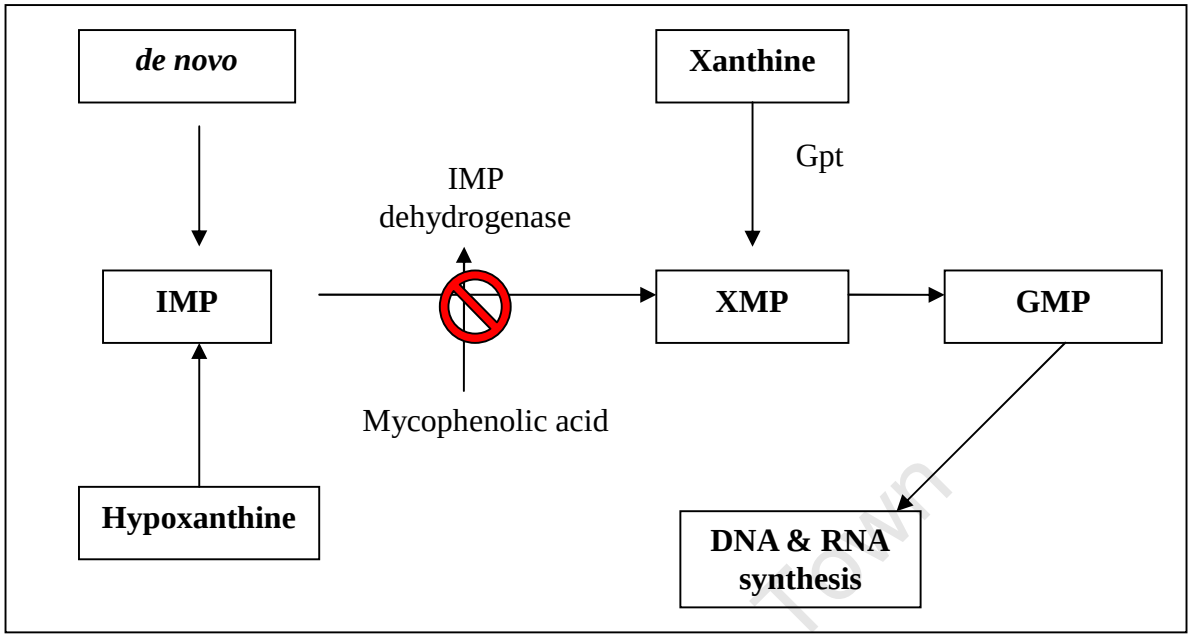

Figure 3.2 *De novo* guanine nucleotide synthesis pathways and the rescue of guanine nucleotide synthesis from mycophenolic acid inhibition by xanthine-guanine phosphoribosyltransferase (Gpt). Mycophenolic acid inhibits inosine monophosphate dehydrogenase and prevents the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP). Gpt utilizes xanthine as an alternative substrate to synthesize XMP, which subsequently gets converted to Guanosine monophosphate (GMP), an important precursor for DNA and RNA synthesis (Falkner & Moss, 1988; Stasolla & Thorpe, 2004)

Reporter genes such as *E.coli* β-galactosidase (β-gal) (An *et al.*, 1982) and β-glucuronidase (GUS) (Jefferson *et al.*, 1986) can enable the selection of recombinant poxviruses by colour. Staining the virus infected cells with colourless substrates of these enzymes results in a blue colour change due to the activity of the enzymes, thus clearly distinguishing the recombinant poxvirus infected cells from the wild-type virus infected cells. Alternatively, a fluorescent reporter gene such as the Green fluorescent protein (GFP) gene can also be used to distinguish the recombinant from wild-type virus with the aid of a fluorescence microscope (Cheng *et al.*, 1996).

This chapter describes the construction of a recombinant LSDV, rLSDV-Grttn, expressing HIV-1 subtype C polyprotein Grttn (Gag, RT, Tat and Nef). The cloning procedures for constructing the transfer vector carrying Grttn are shown in Figures 3.4, 3.8 and 3.10, and the sequence of events in the construction of rLSDV-Grttn are depicted in Figure 3.3. Experiments were performed to confirm the expression of Grttn and to assess the purity of the final recombinant LSDV. The immunogenicity of rLSDV-Grttn was tested in mice and compared to that of DNA and recombinant MVA, both expressing Grttn. Chapter 4 describes the single and prime-boost

vaccination regimens employed using DNA vaccine, rLSDV-Grttn and rMVA-Grttn.

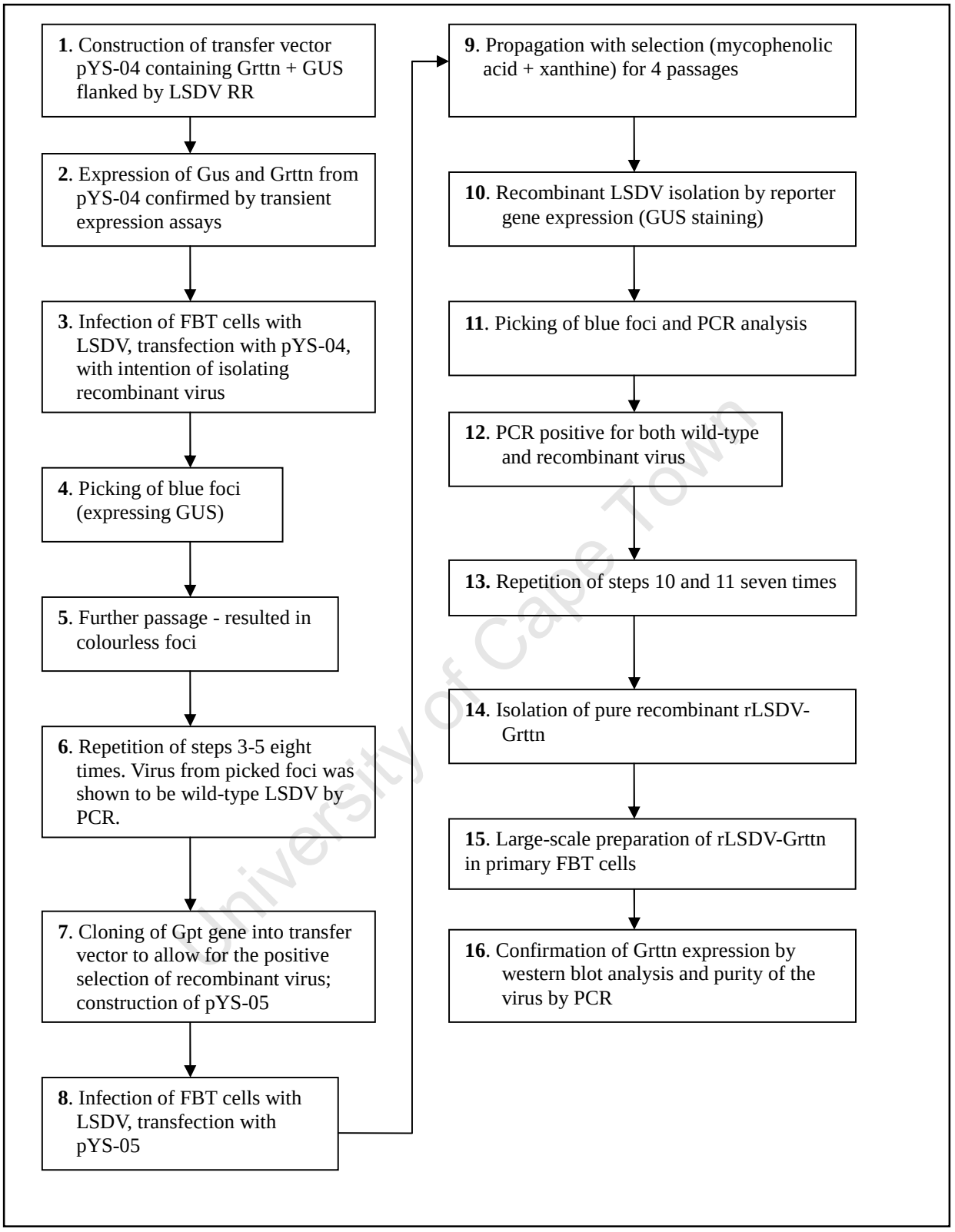

Figure 3.3. Flow chart of experimental progression in the construction of rLSDV-Grttn.

3.2 Construction of Transfer Vectors Required for the Construction of rLSDV-Grttn

3.2.1 Construction of transfer vector pYS-03, containing GUS and VV mH5 promoter flanked by LSDV RR sequences

The plamid pLW-51 (Wang *et al.*, 2004) was used as a backbone for constructing a LSDV transfer vector. The plasmid contains the β-lactamase gene, amp^R, which confers ampicillin resistance. It also contains the reporter gene, β-glucuronidase (GUS) under the control of the VV p11 late promoter which allows recombinant virus to be identified by X-gluc staining for GUS expression (blue coloration). Cloning sites are located downstream of two VV promoters, the strong late synthetic promoter Psyn II and the weaker early/late mH5 promoter. This allows for foreign genes to be expressed from either of these promoters. The GUS gene and multiple cloning sites are flanked by two MVA DNA sequences homologous to the Deletion III region of MVA, MVA flank I and MVA flank II. These flanking regions allow for the insertion of the foreign DNA into MVA genomic DNA by homologous recombination. To modify this plasmid into a LSDV transfer vector, the MVA flanking sequences were replaced with LSDV flanking sequences homologous to the non-essential LSDV ribonucleotide reductase gene. The cloning steps involved are depicted in Figure 3.4.

The two LSDV flanking sequences, LSDV flank I (LFI, 508 bp) and LSDV flank II (LFII, 552 bp), were successfully amplified from LSDV genomic DNA by PCR (Figure 3.5) using the LFI and LFII forward and reverse primers (Table 2.1). In the primer design, restriction enzyme sites *Eco*RI and *Bss*HII were included in the 5' ends of LFI forward and LFI reverse primers respectively. *Pst*I and *Hin*dIII were included in the 5' ends of LFII forward and LFII reverse primers respectively. These restriction enzyme sites were included to allow for the cloning of LFI and LFII into the equivalent restriction enzyme sites of pLW-51, replacing MVA flanks I and II respectively.

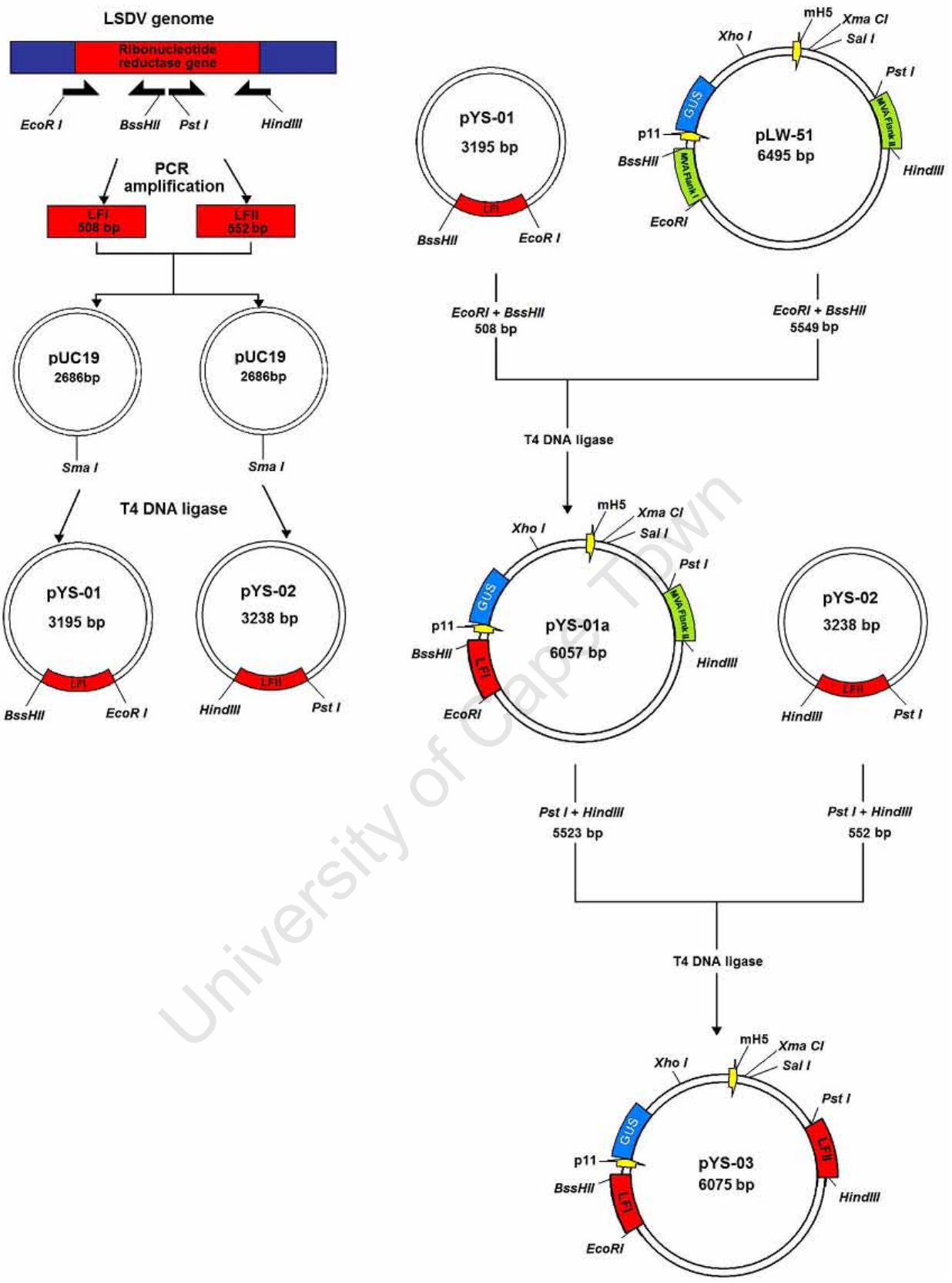

Figure 3.4 Schematic representation (not to scale) of the cloning procedures followed in the construction of pYS-03. LSDV flanking sequences, LFI and LFII, homologous to LSDV ribonucleotide reductase gene, were amplified from LSDV DNA and cloned into pUC19. MVA flank I and II sequences from pLW-51 were replaced sequentially with LFI and LFII excised from pYS-01 and pYS-02 respectively.

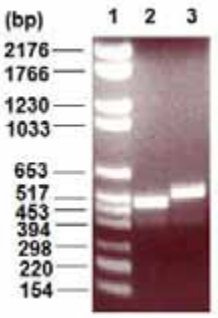

Figure 3.5 Agarose gel electrophoresis of PCR products amplified from LSDV genomic DNA, corresponding to flanking sequences LFI (lane 2, 508 bp) and LFII (lane 3, 552 bp), homologous to the ribonucleotide reductase gene. DNA molecular weight marker VII (Roche, Germany) is in lane 1 with relevant sizes indicated to the left.

Purified LFI and LFII were successfully cloned into the *Sma*I site of pUC19 by blunt-end ligation followed by transformation of competent cells. Recombinant plasmids containing LFI (pYS-01) and LFII (pYS-02) were identified by restriction enzyme analysis and the sequences of the inserts were confirmed by DNA sequencing using pUC universal primers (M13 forward and reverse primer) (Figure 3.6A and B).

A. LFI DNA sequence

GAATTCATGGTATAAAATAAAATGGAACCAATTCTTAAAGAAACATCTTCAAGATTTGTAGTTTT
TCCTATATTATATAAAGAAATATGGGTAATGTATAAAAAAGCTGTCGCTAGTTTTTGGACAGTTG
ATGAAGTAGATCTTTCTAAGGATTTAGCCGATTGGAAAAAACTCAACAATGAAGAGCAATACTTT
ATTAAAAACATATTGGCATTTTTTGCGGCTAGTGATGGTATAGTTAATGAAAATTTGGCCGAAAG
ATTTTACTCGGAGGTACAATGGTCAGAAGCTAGATGTTTTTATGGTTTTCAGATAGCTATGGAAA
ACATACACTCTGAAATGTATAGCTTACTTATTGATACATACATTTCAAGTACTAAAGAGAAGGAA
CATTTATTTAATGCTATTGAAACAATGGATTTTATTAAAAAAAAATCTGAATGGGCTAGAAAATG
GATATCAAACAAAAAAGCATCGTTTGGAGAACGATTAATAGCGTTTGGCGCGC

B. LFII DNA sequence

CTGCAGTTGAGGGAATATTCTTTTCCGGATCATTCGCTGCAATATTTTGGTTAAAAAAACGAGGA
TTGATGCCAGGATTAACATTTTCAAACGAACTTATTAGTAGAGATGAGGGCCTTCATTGTGATTT
TGCGTGTTTAATTTTTAAACATCTAAAATATCCTCCTTCTAATGACATTATTACGATTATTATAA
ACGAGGCTGTGTCTATAGAAAAAGAATTTTTAACCAATATAATTCCTGTAAAACTAATAGGTATG
AATTGTGTTTTAATGTCTCAATATATTGAGTTTATTGCTGATAGATTACTATTAGAACTAGGTTG
TGATAAAGTGTTTATGTCAACAAATCCATTTGATTTTATGGAAAATATATCACTGGAAGGTAAAA
CTAACTTCTTTGAAAAAAGAGTTAGTGAATATCAAAAAATGGGCGTTATTTCTAACAAAGACGAT
AATGTTTTTTCGTTAGATGTTGATTTTTAGTTTTTATTGAATATTATAAAGTTGAATAAATAACA
ACTCTTGTTAATTATCTTGAATAACCAAGCTT

Figure 3.6 DNA sequences of LSDV flanking sequences LFI (A) and LFII (B). The restriction enzyme sites of *Eco*RI + *Bss*HII (in LFI) and *Pst*I + *Hin*dIII (in LFII) required for subsequent cloning steps are indicated in red. LFI and LFII forward and reverse primer binding positions are underlined.

LF1, purified from pYS-01 as a *Bss*HII-*Eco*RI restricted DNA fragment (508 bp), and LF2, purified as a *Pst*I-*Hin*dIII restricted DNA fragment (552 bp), were used in the next two cloning experiments to replace the MVA flanking sequences in pLW-51 (see Figure 3.4). pLW-51 plasmid DNA was subjected to *Eco*RI and *Bss*HII restriction enzyme digestion and the DNA fragment (5554 bp) without the MVA flank I sequence was purified from an agarose gel. This DNA fragment was ligated to the purified LFI DNA and used to transform competent cells. The modified pLW-51 containing LFI (pYS-01a) was identified by restriction enzyme analysis. MVA flank II was successfully removed from pYS-01a by *Pst*I and *Hin*dIII restriction enzyme digestion and the DNA fragment without MVA flank II (5538 bp) was purified from an agarose gel. This DNA fragment was then ligated to the purified LFII DNA fragment and used to transform competent cells. The recombinant plasmid containing LFI and LFII (pYS-03) was identified by single and double restriction enzyme analysis using *Eco*RI, *Hin*dIII, *Sal*I and *Xma*CI restriction enzymes. Figure 3.7A is a schematic representation of pYS-03, showing the expected size fragments from single and double restriction enzyme digestions. The expected size DNA fragments were observed after restriction enzyme digestions (Figure 3.7B), confirming the composition of the transfer vector and the presence of the restriction enzyme sites *Xma*CI and *Sal*I required for subsequent cloning of the HIV-1 polyprotein Grttn gene.

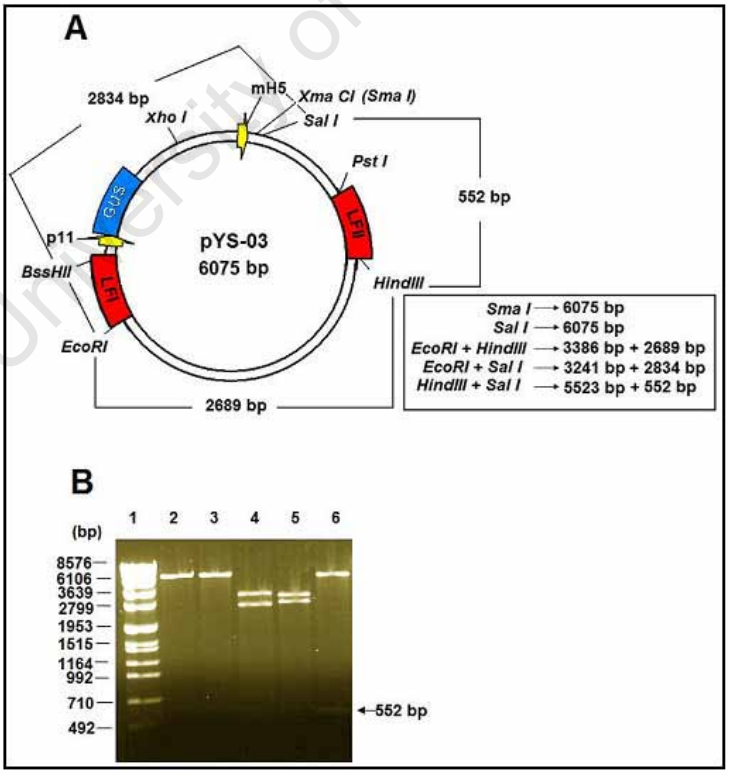

Figure 3.7 Agarose gel electrophoresis of single and double restriction enzyme analysis of pYS-03. (A) Restriction enzyme map (not to scale) showing expected sizes of restriction enzyme digestion products. (B) Electrophoresis of pYS-03 digested with *Sma*I (lane 2, isoschizomer of *Xma*CI), *Sal*I (lane 3), *Eco*RI + *Hin*dIII (lane 4), *Eco*RI + *Sal*I (lane 5) and *Hin*dIII + *Sal*I (lane 6). The arrow on the right points to a faint band of 552bp in lane 6. DNA molecular weight marker VII (Roche, Germany) is in lane 1 with relevant sizes indicated to the left.

3.2.2 Construction of transfer vector pYS-05 containing HIV-1 polyprotein Grttn and *E.coli* gpt inserted into pYS-03

3.2.2.1 Construction of pYS-04

In order to construct a recombinant LSDV expressing HIV-1 polyprotein Grttn, the DNA encoding Grttn was cloned into the LSDV transfer vector pYS-03. The DNA cloning procedure is depicted in Figure 3.8. The DNA fragment containing Kozak-Grttn (3707bp) was successfully excised from pMosBlueGrttn plasmid by *Sal*I and *Xma*CI restriction enzyme digestion, purified from an agarose gel and cloned into the *Sal*I and *Xma*CI restriction enzyme sites of pYS-03, downstream of the VV mH5 promoter. The resulting plasmid construct, pYS-04, containing the DNA encoding Grttn under the control of the mH5 promoter, flanked by LFI and LFII, was confirmed to be correct by restriction enzyme analysis (results not shown) and DNA sequencing from the *Eco*RI site of LF1 to the *Hin*dIII site of LFII (Figure 3.9). The expression of GUS and Grttn was confirmed using transient expression assays (results not shown).

pYS-04 was transfected into LSDV infected FBT cells in an attempt to produce recombinant LSDV expressing Grttn. LSDV-induced foci exhibiting GUS activity (blue foci) were observed and picked. However, upon further passaging in FBT cells, these foci reverted to being colourless. The initial blue foci may have resulted from transient expression of GUS from residual transfer plasmid in the virus-infected cells. After eight transfections with no recovery of recombinant LSDV a positive selection system was employed for isolating recombinant LSDV.

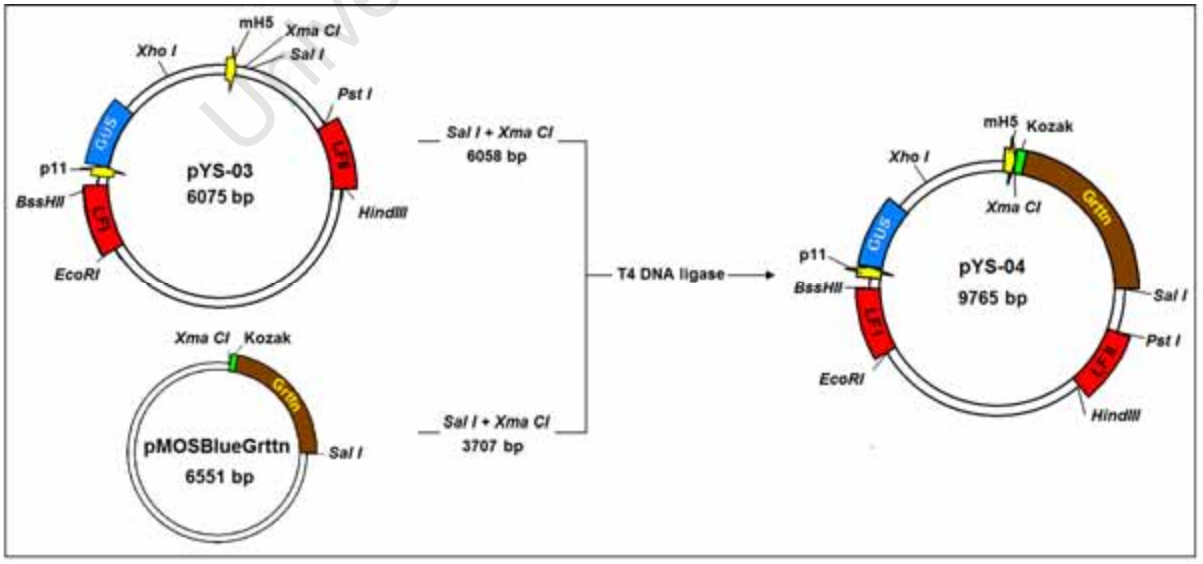

Figure 3.8 Schematic representation (not to scale) of the cloning procedure followed in the construction of pYS-04. Grttn with upstream Kozak sequence was excised from pMOSBlueGrttn and cloned into pYS-03 downstream of the VV mH5 promoter.

Figure 3.9 Continued to the next page

GCAAGCTGAACTGGACCAGCCAGATCTACCCCGGCATCAAGGTGCGGCAGCTGTGCAAGCTGCTGCGGGGCACCAAGGCCCTGA
CCGACATCGTGCCCCTGACCGAGGAGGCCGAGCTGGAGCTGGCCGAGAACCGGGAGATCCTGAAGGAGCCCGTGCACGGCGTGT
ACTACGACCCCAGCAAGGACCTGATCGCCGAGATCCAGAAGCAGGGCGACGACCAGTGGACCTACCAGATCTACCAGGAGCCCT
TCAAGAACCTGAAAACCGGCAAGTACGCCAAGCGGCGGACCACCCACACCAACGACGTGAAGCAGCTGACCGAGGCCGTGCAGA
AGATCAGCCTGGAGAGCATCGTGACCTGGGGCAAGACCCCCAAGTTCCGGCTGCCCATCCAGAAGGAGACCTGGGAGATCTGGT
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GCTACGGCCGCAAGAAGCGCCGCCAGCGCCGCAGCACCCCGCCCAGCAGCGAGGACCACCAGAACCCCATCAGCAAGCAGCCCC
TGCCCCAGACCCGCGGCGACCCCACCGGCAGCGAGGAGAGCAAGAAGAAGGTGGAGAGCAAGACCAAGACCGACCCCTTCGACT
GCAAGTACTGCAGCTACCACTGTCTGGTGTGCTTCCAGACCAAGGGCCTGGGCATCTCCTACGGGCGCAAGAAACGGATGGAGC
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GAAAAAGAATTTTTAACCAATATAATTCCTGTAAAACTAATAGGTATGAATTGTGTTTTAATGTCTCAATATATTGAGTTTATT
GCTGATAGATTACTATTAGAACTAGGTTGTGATAAAGTGTTTATGTCAACAAATCCATTTGATTTTATGGAAAATATATCACTG
GAAGGTAAAACTAACTTCTTTGAAAAAAGAGTTAGTGAATATCAAAAAATGGGCGTTATTTCTAACAAAGACGATAATGTTTTT
TCGTTAGATGTTGATTTTTAGTTTTTATTGAATATTATAAAGTTGAATAAATAACAACTCTTGTTAATTATCTTGAATAACCAA
GCTT

Figure 3.9 DNA sequence of pYS-04 from the *Eco*RI site of LFI to the *Hin*dIII site of LFII. Restriction enzyme sites involved in the cloning steps are underlined. LFI (red), LFII (green), GUS (pink), and Grttn (blue) sequences are colour-coded.

3.2.2.2 Cloning of Positive Selection Gene Gpt into pYS-04

Escherichia coli xanthine-guanine phosphoribosyl transferase (Gpt) was used as the positive selection gene for recovery of recombinant LSDV. The DNA cloning procedure for introducing gpt into the transfer vector is depicted in Figure 3.10.

The DNA fragment containing Gpt and the upstream p7.5 promoter (2.1 kb) was successfully excised from pGPT07/14 plasmid DNA by *Eco*RI restriction enzyme digestion and purified from an agarose gel. The Gpt fragment was blunt ended and cloned into the blunt ended *Xho* I restriction enzyme site in pYS-04. The resulting plasmid pYS-05 was subjected to restriction enzyme analysis, PCR and DNA sequencing. The presence of LSDV flanking sequences, positive selection gene Gpt and HIV-1 polyprotein gene Grttn was successfully demonstrated by PCR (Figure 3.11) using primer pairs listed in Table 2.1. The sequencing data revealed no mutations or errors in Grttn, LFI, LFII or Gpt.

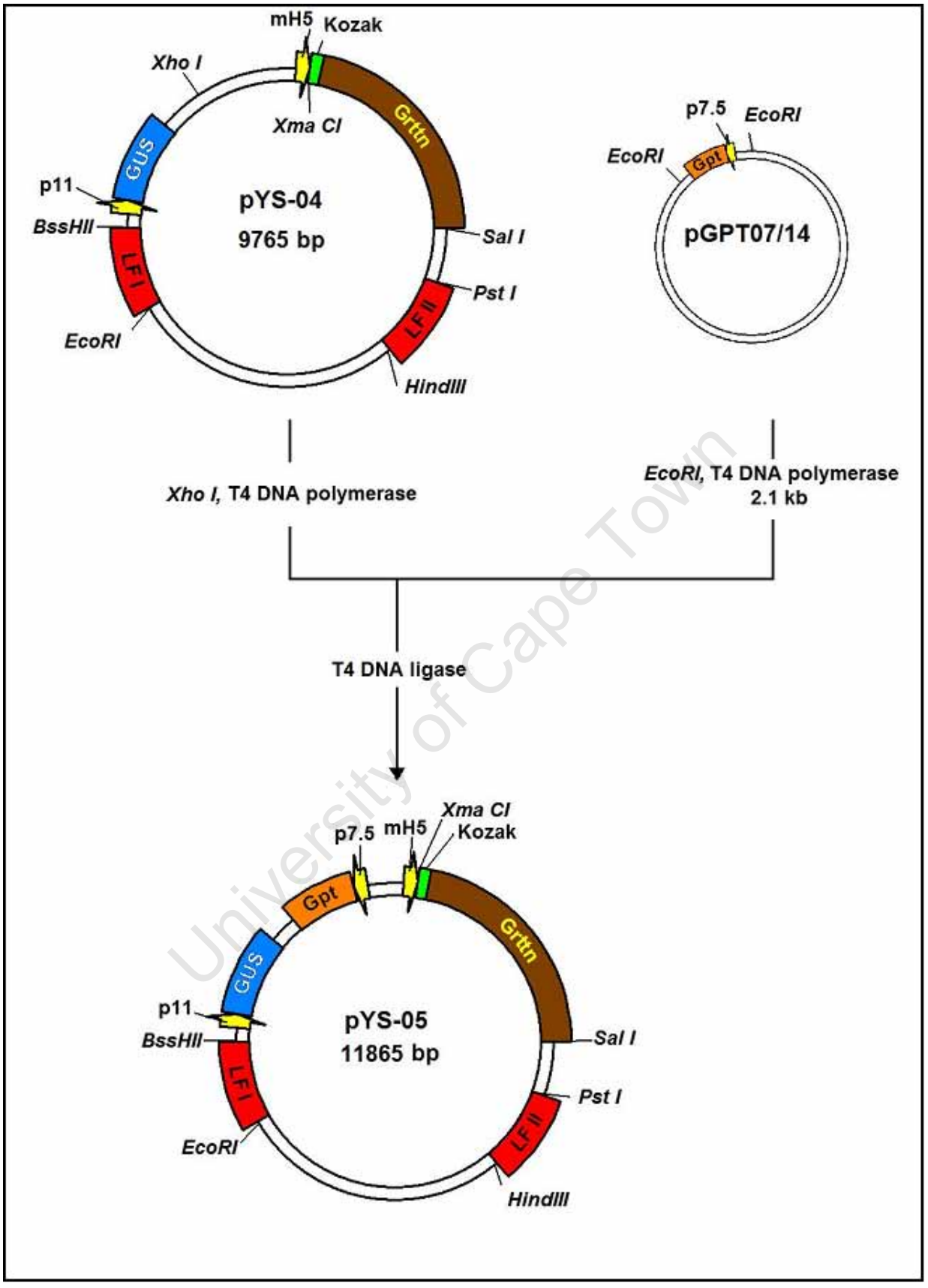


Figure 3.10 Schematic representation (not to scale) of the cloning procedure in the construction of pYS-05. *E.coli* Gpt and the upstream VV p7.5 promoter was excised from pGPT 07/14 using *Eco*RI, blunt-ended and cloned into *Xho*I-digested, blunt-ended pYS-04.

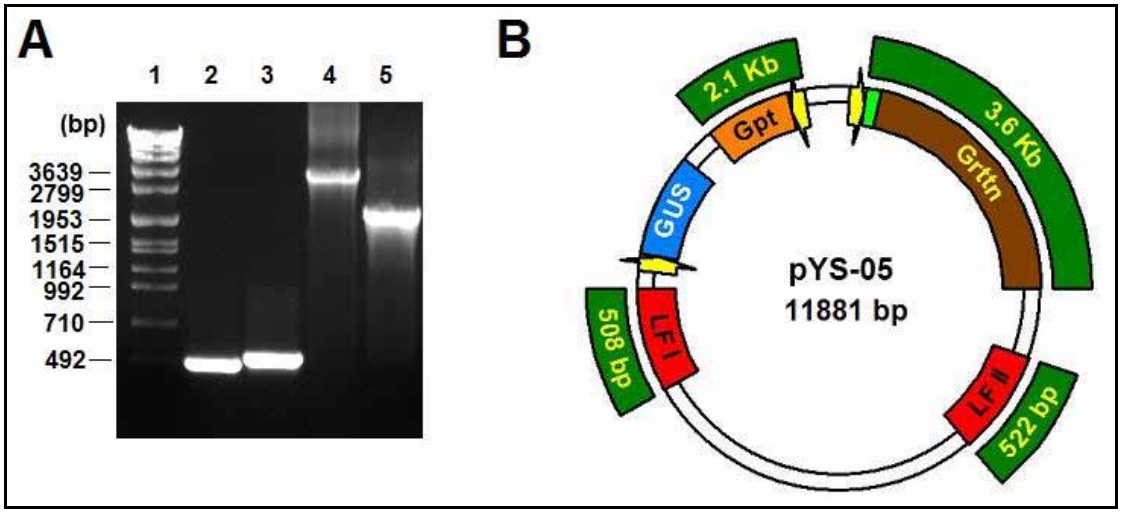


Figure 3.11. Confirmation of composition of pYS-05 by PCR. A**)** Agarose gel electrophoresis of PCR products amplified from pYS-05. LFI (lane 2, 508 bp), LFII (lane 3, 552 bp), Grttn (lane 4, approximately 3.6 kb) and Gpt (lane 5, approximately 2.1 kb) were amplified from pYS-05 using primers listed in table 2.1. DNA molecular weight marker VII (Roche, Germany) is in lane 1 with relevant sizes indicated to the left. B) Schematic representation of pYS-05 (not to scale) showing expected sizes and positions of PCR products ( ).

3.3 Confirmation of VV Promoter Activity and HIV-1 Polyprotein Grttn Expression from pYS-05

It was important to demonstrate the ability of LSDV to recognize the VV promoters in pYS-05 and express GUS and Grttn. Transient assays were performed to detect the expression of GUS and Grttn from pYS-05 in LSDV infected FBT cells.

The transient expression of GUS was detected by a visible blue colour in the cells and agarose overlay and was only detected in LSDV infected, pYS-05 transfected FBT cells (Figure 3.12A). GUS expression was not detected in any of the negative controls which included LSDV infected FBT cells (Fig 3.12B), pYS-05 transfected (uninfected) FBT cells (Figure 3.12 C) and untreated FBT cells (Figure 3.12D). This shows that expression is only achieved in the presence of both virus and plasmid, indicating that LSDV recognizes the late VV p11 promoter.

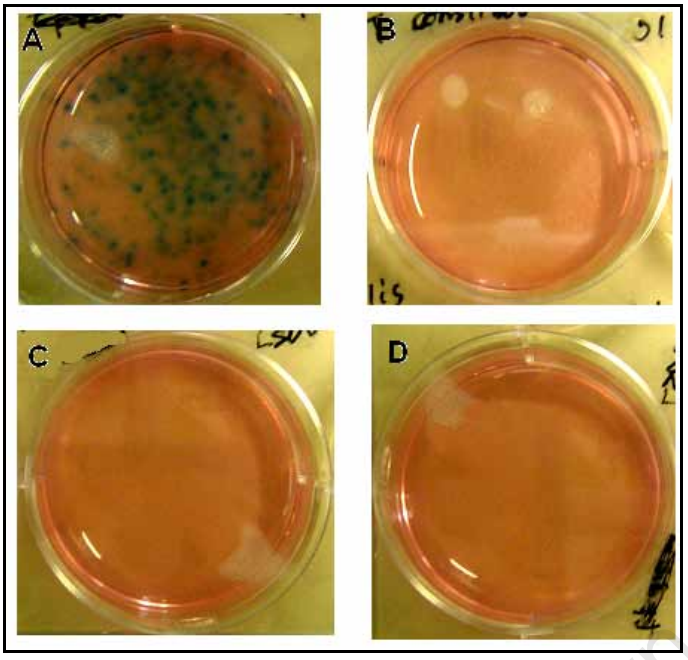


Figure 3.12 Transient expression of GUS. FBT cells were treated with X-gluc and visualized 6 hours later. A) LSDV infected, pYS-05 transfected cells; B) LSDV infected cells; C) pYS-05 transfected cells; and D) uninfected cells.

Transient expression of p24 (in the gag part of Grttn) under the control of the VV mH5 promoter was detected by immunofluorescence using sheep anti-p24 antibodies and FITC-conjugated anti-sheep immunoglobulin. The transient expression of p24 was only detected in LSDV infected, pYS-05 transfected FBT cells (Fig 3.13A) and not in any of the negative controls. The negative controls included LSDV infected FBT cells (Figure 3.13B), pYS-05 transfected FBT cells (Figure 3.13C) and untreated FBT cells (Figure 3.13D). Again, this shows that expression is specifically directed from the pox promoter (early/late mH5 promoter) in LSDV- infected cells transfected with pYS-05.

The transient expression of full length Grttn (150 kDa) was confirmed by western blot analysis and only detected in lysates from FBT cells both infected with LSDV as well as transfected with pYS-05 (Figure 3.14, lane 4). The size (150 kDa) of the protein detected corresponded to the protein expressed in the positive control, pTHgrttnC transfected HEK239 cells (Figure 3.14, lane 2). Expression of the same protein was not detected in the negative controls which included LSDV infected FBT cells, pYS-05 transfected FBT cells and uninfected FBT cells (Figure 3.14, lanes 5-7 respectively).

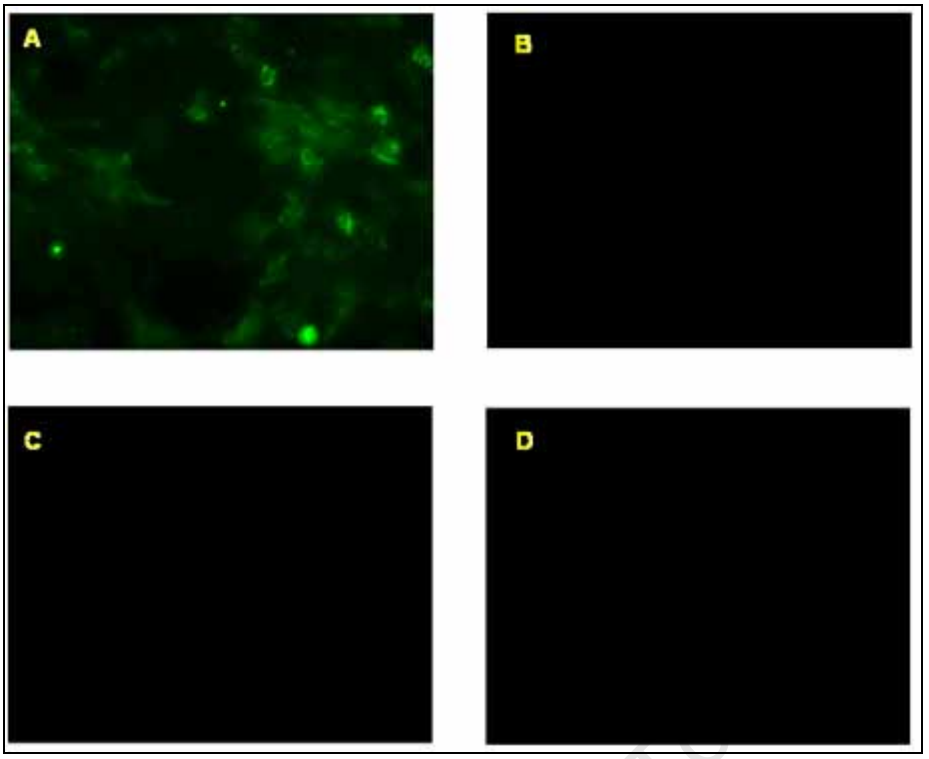


Figure 3.13. Immunofluorescence assay to detect transient expression of p24. FBT cells were treated with p24 (Gag) specific sheep antibody, followed by FITC conjugated anti-sheep immunoglobulin. p24 expression was visualized as green fluorescent signals. A) LSDV infected, pYS-05 transfected cells; B) LSDV infected FBT cells; C) pYS-05 transfected FBT cells; and D) uninfected FBT cells.

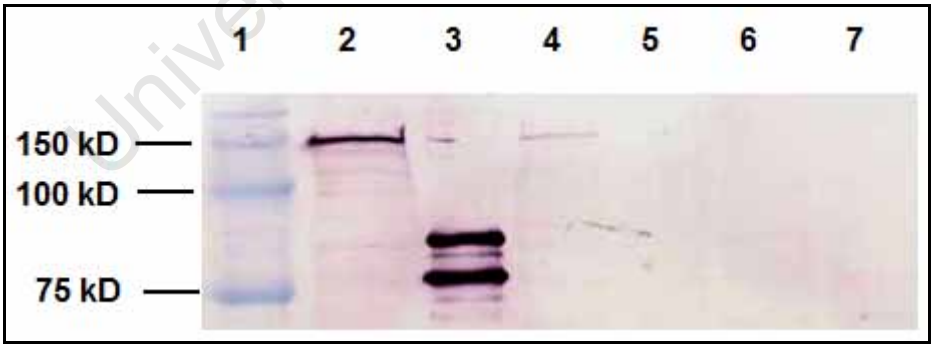


Figure 3.14 Western blot analysis on transient expression of Grttn. Western blot of lysates of pTHgrttnC transfected HEK239 cells (lane 2), LSDV infected and pYS-05 transfected FBT cells (lane 4), LSDV infected FBT cells (lane 5), pYS-05 transfected FBT cells (lane 6) and uninfected FBT cells (lane 7), treated with RT specific sheep antibody, followed by alkaline phosphatase conjugated anti-sheep immunoglobulin antibody. RT protein standard is in lane 3 and Kaleidoscope Precision Plus Protein prestained standards (Bio-Rad, USA) are in lane 1 with sizes indicated to the left.

3.4 Construction of rLSDV-Grttn

The recombinant LSDV expressing HIV-1 subtype C polyprotein Grttn was successfully produced in the following way. FBT cells were infected with wild-type LSDV and then transfected with plasmid pYS-05. Infected cells were harvested and the released virus was passaged four times on fresh FBT cells in the presence of selection medium. This was to select for recombinant LSDV expressing gpt and suppress the growth of wild-type LSDV with mycophenolic acid.

Virus harvested from the lysed cells was serially diluted ten-fold and FBT cells were infected with different dilutions of the virus isolated. In this way distinct foci could be dispersed for picking. After GUS staining blue foci, representing recombinant virus, were picked. Figure 3.15 shows recombinant LSDV-infected FBT cells after GUS staining (A) and a single focus (400 x magnification) produced by recombinant LSDV expressing GUS (B). Blue foci were plaque purified eight times in FBT cells with selection medium, before a pure recombinant focus was chosen for large scale preparation.

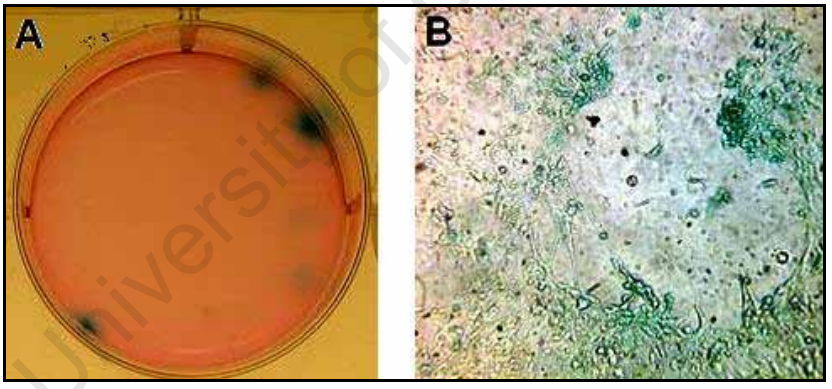


Figure 3.15 GUS expression in rLSDV-Grttn infected FBT cells. A) Cells with agarose overlay stained with X-gluc. B) Focus of recombinant virus expressing GUS (400X).

3.5 Large Scale Preparation of rLSDV-Grttn and Titration

Recombinant LSDV (rLSDV-Grttn) was propagated in primary FBT cells under positive selection (gpt) conditions. Although the growth of LSDV had been demonstrated to be better in primary foetal bovine or lamb testes cells compared to bovine cell lines, it was still difficult to propagate the rLSDV-Grttn to high titres for subsequent animal inoculations (10^7 to 10^8 ffu/ml). Furthermore the FBT cells could only be passaged 6 to 7 times. Thereafter frozen FBT cells had

to be seeded for further LSDV propagation. Sixteen to twenty (175 cm^2) flasks of FBT cells were prepared for each round of passage. Over the duration of the project, FBT primary cell lines were prepared seven times due to the fast depletion of the frozen cell stock.

Although the preliminary growth curve experiment (result not shown) indicated that the titre of the virus peaked at 72 hours post infection, and rLSDV-Grttn was harvested at this time point after each passage, rLSDV-Grttn still had to be propagated in large volumes (virus infected cell lysates reached 1-1.5 litres), before a high enough titre could be obtained for mice inoculations. Mycophenolic acid in the selection medium might have had a negative effect on FBT cell growth, and might have indirectly affected the growth of the recombinant LSDV. rLSDV-Grttn was propagated, concentrated and purified from the cell debris by centrifugation through a 36% sucrose cushion.

Although LSDV replicates better in primary FBT cells compared to MDBK cells (Wallace 1994), LSDV-induced CPE on MDBK cells (ridges) were more visually distinct compared to the LSDV induced foci in FBT cells. Therefore, the rLSDV-Grttn stock was titrated on MDBK cells. The titre of rLSDV-Grttn was determined by immunostaining with anti-RT antibody. Figure 3.16A shows a positive focus of infection. The virus titre from an initial volume of 1 litre of cell lysate was determined to be 8 x 10^7 ffu which was concentrated to a final volume of 1.5 ml (5.3 x 10^7ffu/ml).

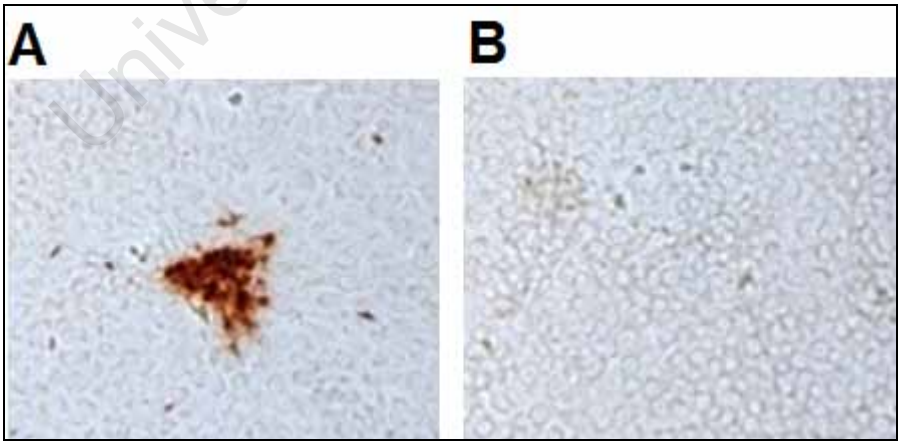


Figure 3.16 Titration of rLSDV-Grttn by immunostaining. rLSDV-Grttn (A) and wtLSDV (B) infected MDBK cells were treated with RT specific sheep antibody, followed by peroxidase conjugated anti-sheep antibody. The titre of rLSDV-Grttn was determined by counting RT expressing foci as seen in the Figure (100X magnification).

3.6 Assessment of the Purity of rLSDV-Grttn

To ensure the rLSDV-Grttn isolated was free from any wild-type LSDV, PCR primers were designed to amplify specific DNA fragments from rLSDV-Grttn and wild-type LSDV. The positions of primer binding are schematically represented in Figure 3.17A. rLSDV-Grttn or wild-type LSDV infected FBT cells were lysed and the lysates were used as the DNA template in the PCR. The simultaneous presence of both 1.2 and 1.4kb DNA products would indicate that there was wild-type LSDV contamination in the rLSDV-Grttn stock. Both recombinant (1.2kb) and wild-type (1.4kb) PCR products were detected in lysates of cells infected with an earlier passage (passage 4) of rLSDV-Grttn, demonstrating that the PCR assay could effectively detect wild-type and recombinant virus in a single reaction (Figure 3.19.B, lane 5). Only the recombinant DNA product (1.2 kb) was amplified in the final rLSDV-Grttn stock (Fig 3.17B, lane 4) indicating that the rLSDV-Grttn isolated was free from any wild-type LSDV.

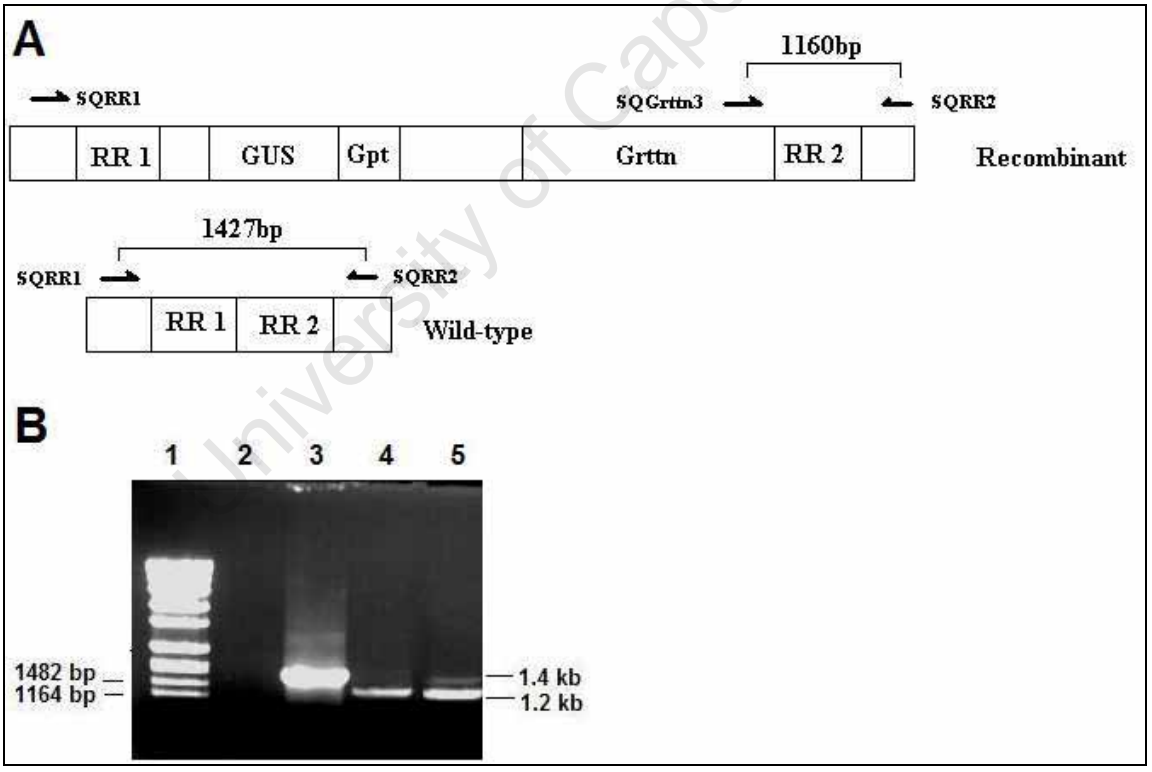


Figure 3.17 Detection of wild-type LSDV and rLSDV-Grttn by PCR. A) Schematic representation of primer binding sites and the respective sizes of the DNA products amplified from recombinant and wild-type LSDV. B) Agarose gel showing PCR products from the lysates of FBT cells (lane 2), LSDV infected FBT cells (lane 3) and rLSDV-Grttn infected FBT cells (lane 4). Lane 5 shows the products from an earlier passage of rLSDV-Grttn (passage 4). DNA molecular weight marker VII (Roche, Germany) is in lane 1 with relevant sizes indicated to the left.

3.7 Full Length HIV-1 Polyprotein Grttn Expression from rLSDV-Grttn

The expression of full length Grttn was confirmed using western blot analysis with HIV-1 Gag- and RT-specific antibodies. FBT cells were infected with rLSDV-Grttn, wild-type LSDV or left uninfected. These FBT cells were then lysed and subjected to PAGE and Western blot analysis. Gag and RT protein standards and a lysate of BHK-21 cells infected with recombinant MVA known to express Grttn and HIV-1 gp150 (SAAVI-MVA-C) were included as positive controls.

Expression of protein with a molecular weight of 150 kDa, corresponding to the size of Grttn was detected in rLSDV-Grttn infected FBT cell lysates (Figure 3.18A, lane 4; Figure 3.18B, lane 3). Expression of Grttn was absent from wild-type LSDV infected FBT cell lysates (Figure 3.18A - lane 5; B – lane 4). This experiment confirmed that Grttn was expressed in its entirety from rLSDV-Grtn.

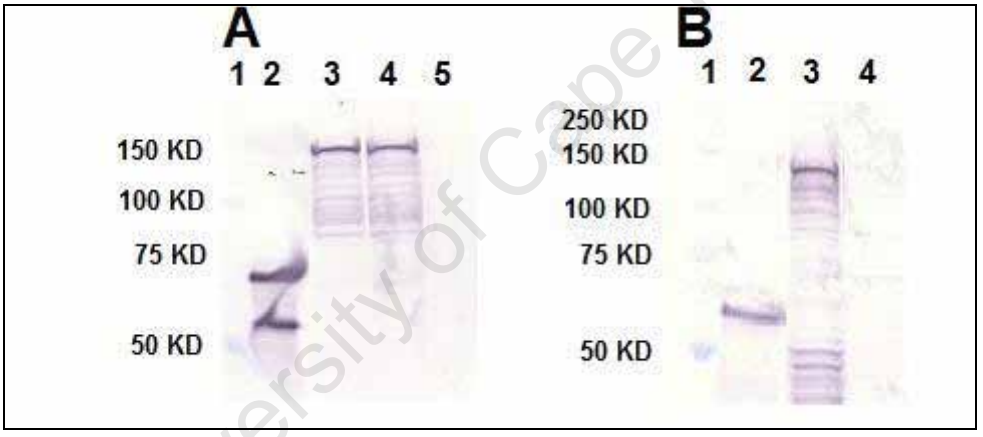


Figure 3.18 *In vivo* expression of Grttn in rLSDV-Grttn infected FBT cells. Lysates were subjected to western Blot analysis using RT-specific (A) or p24-specific (B) sheep antibody, followed by alkaline phosphatase conjugated anti-sheep antibody. A). Western blot of lysates of SAAVI-MVA infected BHK-cells (lane 3), rLSDV-Grttn infected FBT cells (lane 4) and WtLSDV infected FBT cells (lane 5). Lane 2 contains RT protein standard. Protein molecular weight standards are in lane 1 with sizes indicated to the left B) Western blot of lysates from rLSDV-Grttn infected FBT cells (lane 3) and WtLSDV infected FBT cells (lane 4). Gag protein standard is in lane 2 and Kaleidoscope Precision Plus Protein prestained standards (Bio-Rad, USA) is in lane 1 with sizes indicated to the left.

3.8 Discussion

In this part of the study, a LSDV transfer vector containing genes encoding HIV-1 polyprotein Grttn, reporter protein GUS and positive selection enzyme Gpt, all flanked by DNA sequences homologous to LSDV ribonucleotide reductase gene, was successfully constructed. In addition

to confirming the construction of the transfer plasmid by restriction enzyme analysis and DNA sequencing, the expression of the foreign genes inserted was successfully confirmed using transient expression and detection assays in LSDV infected, transfer plasmid transfected FBT cells. The reporter gene, Gus, activity was confirmed through GUS staining and the expression of Grttn was detected by immunofluorescence and western blot analysis.

Recombinant LSDV expressing the HIV-1 subtype C polyprotein Grttn (rLSDV-Grttn) was successfully constructed. Although other recombinant poxviruses such as vaccinia virus and MVA have been produced with relative ease (Mackett *et al.*, 1982; Panicali *et al.*, 1983), the particular recombinant desired in this study was difficult to isolate. Initially the construction of the rLSDV-Grttn was attempted without the aid of a positive selection gene. All attempts at isolating the rLSDV with the aid of only the reporter gene GUS failed. The positive selection gene, gpt, was introduced to suppress wild-type LSDV growth and increase the probability of isolating the recombinant LSDV. This approach was successful and rLSDV-Grttn was successfully constructed and isolated. However, it is acknowledged here that repeated sequences should have been introduced into the transfer vector flanking the Gpt and Gus genes in order to remove these genes from the final recombinant virus.

rLSDV-Grttn was viable in FBT cells and it could be propagated to the required experimental titre (approximately 10^8 ffu/ml). The CPE induced by rLSDV-Grttn showed no difference from that induced by the wild-type LSDV. The construction of this recombinant affirms the ability to use the ribonucleotide reductase gene as an insertion site for the generation of stable recombinant LSDV (Aspden *et al.*, 2002; Aspden *et al.*, 2003). Expression of the full-length Grttn protein from the VV mH5 promoter was demonstrated by western blot analysis. This further supports the finding that promoter sequences are highly conserved within the *Chordopoxviridae* subfamily (Boyle, 2007; Prideaux *et al.*, 1990). Stable expression of Grttn was detected after 17 passages of rLSDV-Grttn in FBT cells, under positive selection conditions. The lack of contaminating wtLSDV in the rLSDV-Grttn stock was demonstrated by PCR.

rLSDV-Grttn was constructed as a potential HIV-1 vaccine candidate. Within our department a recombinant MVA expressing Grttn (rMVA-Grttn) has been constructed (N. Johnson, unpublished). The same promoter was used for the control of Grttn expression in the two vaccine candidates. Ideally, for comparative purposes, it would be desirable to use the equivalent sites of insertion for the two vaccine candidates. However, Grttn was inserted into the Del III region of MVA and such a region is absent from the LSDV genome, so Grttn could not be inserted into the equivalent region in LSDV. For a better comparison of the two vaccine

vectors, rMVA-Grttn could have been reconstructed with Grttn inserted into the ribonucleotide reductase gene of MVA, but this was beyond the scope of this project.

Another non-essential gene, the thymidine kinase gene, has been used for the insertion of foreign genes into LSDV (Wallace *et al.*, 2006; Wallace *et al.*, 2007; Wallace & Viljoen, 2005). Although these (TK and RR) insertion sites were demonstrated to be non-essential, there could still be a difference in growth or fitness of the recombinant virus generated using different insertion sites. The effect of disrupting the ribonucleotide reductase or the thymidine kinase genes on LSDV growth or ability to express foreign protein has not been evaluated. However, disruption of either of these genes in VV leads to mild attenuation *in vivo* (Buller *et al.*, 1985; Child *et al.*, 1990; Taylor *et al.*, 1991a).

Because our intention was to compare LSDV to MVA as a vaccine vector, we used the same promoter (mH5) and antigen (Grttn) which was used in the construction of rMVA-Grttn. (Wyatt *et al.*, 2008) have shown that higher levels of *in vitro* antigen expression is positively correlated with better immunogenicity of rMVA vaccine in mice. Therefore the immunogenicity of a vaccine candidate is likely to be affected by the level of antigen expression in vaccinated individuals. However, the use of a strong promoter may reduce the stability of the recombinant poxvirus. Testing other poxvirus promoters could be considered to optimise the level of antigen expression, without compromising the stability of the recombinant LSDV.

The HIV-1 antigens chosen in this study include important epitopes across HIV-1 subtype C capsid protein, reverse transcriptase, Tat and Nef, however, the glycoprotein (Env) was missing from rLSDV-Grttn. Because this is the first attempt to evaluate LSDV as a HIV-1 vaccine vector, the study was designed to be a "proof of concept" evaluation of the potential of LSDV as a HIV-1 subtype C vaccine vector. Future candidate rLSDV vaccines could include Env epitopes to optimize the coverage across the HIV-1 subtype C genome. HIV-1 subtype C was chosen because it is the predominant subtype found in Sub-Saharan Africa, which is also the region worst affected by the HIV-1 pandemic. The use of LSDV as a vaccine vector in this area would be considered acceptable because LSD is endemic in the area. In addition the LSDV strain used is the vaccine strain given to cattle in the region (Kara *et al.*, 2003). However the introduction and use of LSDV in non-LSD affected or exposed regions (predominated by non-subtype C HIV-1) may encounter resistance from local regulatory bodies.

rLSDV-Grttn constructed in this project is a valuable tool for evaluating the immunogenicity of a recombinant LSDV vaccine for HIV-1 in animal models, and this will be the subject of the

next chapter. Unfortunately, there are a number of drawbacks of this particular construct for use in humans. It is not desirable to have reporter or selection genes in the final recombinant. The deletion of undesirable foreign genes such as the reporter gene and positive selection gene could be achieved by simply engineering into the transfer vector a repeated sequence flanking the genes which need to be deleted. After isolating a pure recombinant containing the marker gene, this virus would then be further passaged in the absence of selection, and colourless foci could be picked to select for a recombinant containing the foreign gene of interest, but no marker or positive selection genes. The desired recombinant could be identified by PCR analysis.

A major concern is the lack of an approved cell line for manufacture of recombinant LSDV. A recombinant LSDV from a research lab would have to be remade in a GMP compliant facility using approved cell lines and reagents. LSDV grows optimally in bovine primary cells (Wallace, 1994). However these primary cells isolated from bovine tissue have a limit to the number of times that they can be passaged, and fresh cells need to be isolated regularly. The quality control of the cells isolated cannot be maintained easily due to the genetic variability of individual calves and the condition of tissue harvested each time. Most importantly, opportunistic contaminants such as retroviruses and prions, such the causal agent of bovine spongiform encephalopathy, (BSE), may be present in these primary bovine cells (Asher, 1999a; Asher, 1999b), necessitating the screening of cells after each primary cell isolation. Thus the primary FBT cells are unlikely to be approved by the regulatory bodies for the production of rLSDV intended for human use. The use of the ovine testis cell line OA3.Ts for the propagation of capripoxviruses, including LSDV, has been shown to produce comparable titres of virus to that of primary cells (Babiuk *et al.*, 2007). However the cell line will need to be evaluated for its suitability and approved by regulatory agencies for human vaccine production. Propagation of rLSDV in Vero cells, an acceptable cell line for human vaccine production (influenza) has also been demonstrated (Babiuk *et al.*, 2007); however the virus yield is about 100 times less compared to OA3.Ts and primary cells (Babiuk *et al.*, 2007). LSDV can be propagated on the chorioallantoic membranes (CAMs) of embryonated eggs, and this is partly how LSDV was attenuated in the generation of the Neethling vaccine strain (van Rooyen *et al.*, 1969). Eggs are commonly used for the production of vaccine for human use. However, like the Vero cell line, a lower yield of LSDV was obtained in embryonated eggs compared to bovine primary cell lines (Wallace, 1994). The use of animal cell lines and embryonated eggs should be further evaluated and optimized as alternative means of rLSDV production. A recombinant could possibly be made in Vero cells and then grown to high titres on the CAMs of embryonated eggs.

As an alternative means of ensuring the recombinant LSDV is clear of retroviruses or prions,

rLSDV could be reconstructed in a Bacterial artificial chromosome system (BAC) (Cottingham *et al.*, 2008; Domi & Moss, 2002). This will ensure the purity of the rLSDV vaccine stock without numerous plaque purifications in tissue culture. The method involves the manipulation of the poxvirus genome in a BAC system. The entire head to tail poxvirus DNA concatamer intermediate is cloned into a BAC. This allows for the manipulation of poxvirus DNA in *E.coli* through an efficient DNA homologous recombination technique directed by bacteriophage-lambda enzyme (recombineering, http://recombineering.ncifcrf.gov). Because poxvirus DNA is not infectious alone, due to the absence of the early transcription system which is packaged into the virion core of viral particles, transfected recombinant poxvirus genomic DNA will need to be rescued by another poxvirus infecting the cells in order to grow the final construct in cell culture (possibly Vero cells). This rescue virus provides the necessary enzymes to initiate early transcription and replication but should not produce infectious progeny in the cell line which is transfected with recombinant poxvirus DNA. Host range-restricted Avipoxviruses would be suitable for the rescue of rLSDV. Once the desired recombinant LSDV is acquired, it could be propagated to the titre required for vaccine production, possibly on CAMs of embryonated eggs.

In conclusion to this chapter, a viable recombinant LSDV expressing HIV-1 polyprotein Grttn, rLSDV-Grttn, was constructed. The evaluation of the immunogenicity of this vaccine in BALB/C mice is the topic of the next chapter.

Chapter 4 Safety of LSDV as a Vaccine Vector and Immunogenicity of rLSDV-Grttn

4.1 Introduction

The safety and efficacy of a new candidate vaccine are critical aspects which need to be investigated prior to the advancement of the vaccine to clinical trials. Because of the high HIV-1 prevalence in regions such as Sub Saharan Africa (Luthy *et al.*, 2006; Redfield *et al.*, 1987; Rivas *et al.*, 2007; Wyatt *et al.*, 2004) it is important to evaluate the safety of a potential HIV-1 vaccine in both immune-competent and immunocompromised hosts, especially if the vaccine has the potential to be used as a therapeutic vaccine. The safety of MVA in an immunocompromised setting was demonstrated in SCID mice (Wyatt *et al.*, 2004). Previously, the vaccine had been tested in humans and shown to be safe as a vaccine against smallpox (Stickl *et al.*, 1974; Wyatt *et al.*, 2004). The Neethling strain of LSDV has been demonstrated to be non-pathogenic in ruminant and non-ruminant animals, and shown to be host restricted in cell culture (Aspden *et al.*, 2002; Aspden *et al.*, 2003). However, the safety of LSDV has not been investigated in an immunocompromised setting. The present study has addressed this issue by evaluating the safety of the Neethling strain of LSDV (wtLSDV) in two strains of immunocompromised mice with different immunodeficiency phenotypes. CD4 knockout mice, which are deficient in CD4 T cell production, and RAG mice, which are deficient in both mature T and B cells, were vaccinated with wtLSDV and the well being and weight of the mice recorded for 30 days after vaccination.

Pre-clinical evaluation of candidate HIV-1 vaccines relies on *ex vivo* immunogenicity assays, which determine both cellular and humoral immune responses induced by the vaccine. These *ex vivo* assays measure the frequency and functionality of the immune cells by evaluating factors such as cytokine secretion and cell surface molecule expression (Ghanekar & Maecker, 2003; Maecker & Maino, 2003; Zhang *et al.*, 2009). Cytokines released from T cells function both directly and indirectly on the immune activity of T cells (Bevan, 2004; Haynes *et al.*, 1989; Peters *et al.*, 1991; Schroder *et al.*, 2004; Sun *et al.*, 2004). The Th1 cytokines IFN-γ and IL-2 are known to be associated with the activation and maintenance of CD4+ and CD8+ T cells (Bevan, 2004; Boehm *et al.*, 1997; Mach *et al.*, 1996; Schroder *et al.*, 2004; Sun *et al.*, 2004) and are associated with the induction and modulation of cellular immune responses (Abbas *et al.*, 1996; Mosmann *et al.*, 1986), which are important for antiviral immunity (Barker *et al.*, 1995; Clerici *et al.*, 1993; Maggi *et al.*, 1987). IL-2 secretion by CD4+ T cells is known to be associated with growth stimulation, differentiation and survival of antigen-specific cytotoxic T cells (Bevan, 2004; D'Souza *et al.*, 2002; Sun *et al.*, 2004). IFN-γ and IL-2 ELISPOT assays are

used to evaluate the frequency of antigen–specific IFN-γ and IL-2 producing cells (Asmuth *et al.*, 2009; Burgers *et al.*, 2009; Harro *et al.*, 2009; Lalvani & Pareek, 2009; Sadagopal *et al.*, 2005; Shephard *et al.*, 2008). The cytokines produced by antigen-stimulated immune cells are captured by cytokine specific antibody coated onto membranes. Cytokine specific biotinylated antibody is then added and subsequently probed with horse radish peroxidise (HRP) conjugated with Avidin. Spots, which respresent cytokine producing cells, are visualized after HRP substrate incubation. Enumeration of antigen specific cytokine producing cells can be achieved by counting the spots.

Antigen specific cells in the immune cell population can be detected and counted using flowcytometry after binding of fluorochrome conjugated pentramic MHC-peptide complexes to complementary T cell receptors and suitable different fluorescent labeled antibodies (e.g. CD8) to suface receptors. (Almeida *et al.*, 2007; Arora, 2002; Betts *et al.*, 2003; Bridgeman *et al.*, 2009; Brooimans *et al.*, 2008; Chattopadhyay *et al.*, 2008; Cottingham *et al.*, 2006; Shephard *et al.*, 2008). CD44 expression on T cells has been shown to be up-regulated in response to antigen-specific T cell activation (Budd *et al.*, 1987; Haynes *et al.*, 1989; Lesley *et al.*, 1993). High and intermediate levels of expression of CD44 on T cells are associated with a memory T cell phenotype (Budd *et al.*, 1987; Dutton *et al.*, 1998). The effector function of cytotoxic T cells is related to the capacity of these cells to degranulate lytic granules containing perforin and granzymes. The membrane surfaces of these lytic granules are associated with lysosomal-associated membrane proteins (LAMP) such as CD107a/b (Peters *et al.*, 1991). The active degranulation process exposes CD107a/b transiently on the membrane of activated cytotoxic T cells (Peters *et al.*, 1991), allowing CD107a/b to be a marker of degranulation activity of these T cells (Betts *et al.*, 2003; Betts & Koup, 2004; Peters *et al.*, 1991).

This chapter addresses the immunogenicity of rLSDV-Grttn on its own and in different prime-boost vaccination regimens with a DNA vaccine, pVRCgrttnC, and a recombinant MVA vaccine, rMVA-Grttn, in BALB/c mice. Splenocytes from vaccinated mice were used to determine cellular immune responses to Gag and RT. Initially IFN-γ and IL-2 ELISPOT assays were used to enumerate Gag- and RT-specific CD8+ and CD4+ T cells secreting IFN-γ and IL-2. These assays contained splenocytes and peptides with amino acid sequences matching BALB/c CD8 and CD4 T cell epitopes in Gag and RT as stimuli. The level of IFN-γ and IL-2 released into the culture medium by these splenocytes during culture with the Gag and RT CD8+ and CD4+ T cell peptides was evaluated using a cytokine bead array assay and flow cytometry. Pentameric

H-2D^k complexes folded with the Gag peptide AMQMLKDTI and pentameric H-2D^d complexes folded with the RT peptide VYYDPSKDLIA were used to enumerate total HIV-specific CD8+ T cells generated by the heterologous poxvirus vaccination regimens. In addition the functional capacity of the pentameric positive cells was assessed by expression of CD44 and CD107a/b. The humoral responses induced by the heterologous vaccination regimens were evaluated by detecting the presence of Gag- specific antibodies in the sera of immunized mice using a LAVblot I assay.

4.2. Safety of LSDV in Immunocompromised Mice

LSDV has previously been demonstrated to be safe in mice and cattle. However the safety in immunocompromised animals remains to be investigated. Safety of wtLSDV (Neethling strain) was evaluated in two strains of immunocompromised mice, CD4 knockout mice and RAG mice. Two different doses of wtLSDV (10^4 ffu and 10^6 ffu) were tested in each of these strains. Naïve mice and mice vaccinated with PBS served as controls in the experiment. For all the mouse groups no change in their well-being was observed up to 30 days after vaccination with wtLSDV or PBS. They were active and inquisitive when disturbed and their hair coat remained clean and groomed throughout the experiment, indicating no adverse affects of wtLSDV. Mice weights were also recorded daily for 30 days as an indicator of possible pathogenicity of the virus (Figure 4.1). A fluctuation in the weight of the individual mice in each group was observed however a general increase in weight for all mouse groups was seen (Figure 4.1). Prior to the experiment a decision was made to statistically analyse the effect of wtLSDV on mouse weight on day 6, day 11 and day 30 post vaccination. These time points were chosen based on critical times during the development of immune responses to rLSDV-grttn. On these days the percentage change in weight from that pre-vaccination for all mice in a group was calculated (Figure 4.2). To determine any significant effect of wtLSDV vaccination on weight change this data for each group on day 6, day 11 and day 30 was statistically analysed using one-way ANOVA (Figure 4.2).

CD4 knockout mice vaccinated with 1 x10^4 ffu or 1 x 10^6 ffu of wtLSDV did not show any significant changes in weight at days 6 ($p= 0.79$), 11 ($p= 0.55$) and 30 ($p= 0.24$) compared to mice vaccinated with PBS or left unvaccinated (naïve) (Figure 4.2). Similar observations were made with RAG mice at day 6 ($p= 0.18$) and day 30 ($p= 0.16$). The difference in percentage weight changes at day 11 between the 1 x10^4 ffu or 1 x 10^6 ffu of wild-type LSDV inoculated

groups and the negative control groups (PBS-inoculated and naïve RAG) were statistically different (p= 0.04), however the percentage weight change (weight loss) did not occur in a LSDV-titre dependent manner, which may indicate that the significance of differences between the percentage weight change in these RAG mice was questionable (Figure 4.2).

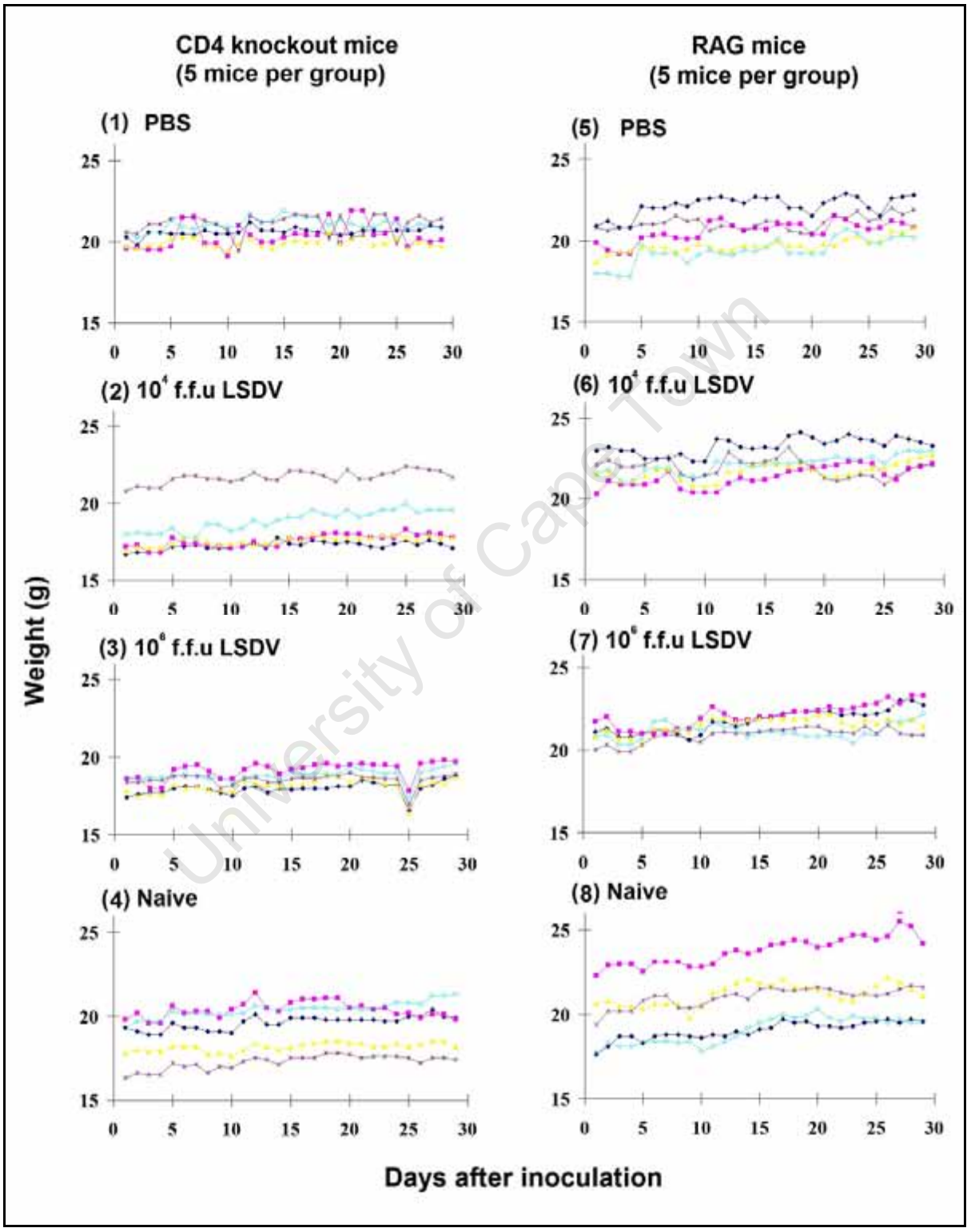


Figure 4.1 Evaluation of the safety of LSDV in immunocompromised mice using daily weights as an indicator. Immunocompromised CD4 knockout (1-4) and RAG (5-8) mice were inoculated with PBS (1 and 5), 10^4 ffu of LSDV (2 and 6), 10^6 ffu of LSDV (3 and 7) or not vaccinated (naïve, 4 and 8). The weight recorded for the 5 individual mice in each group for a period of 30 days after vaccination has been plotted.

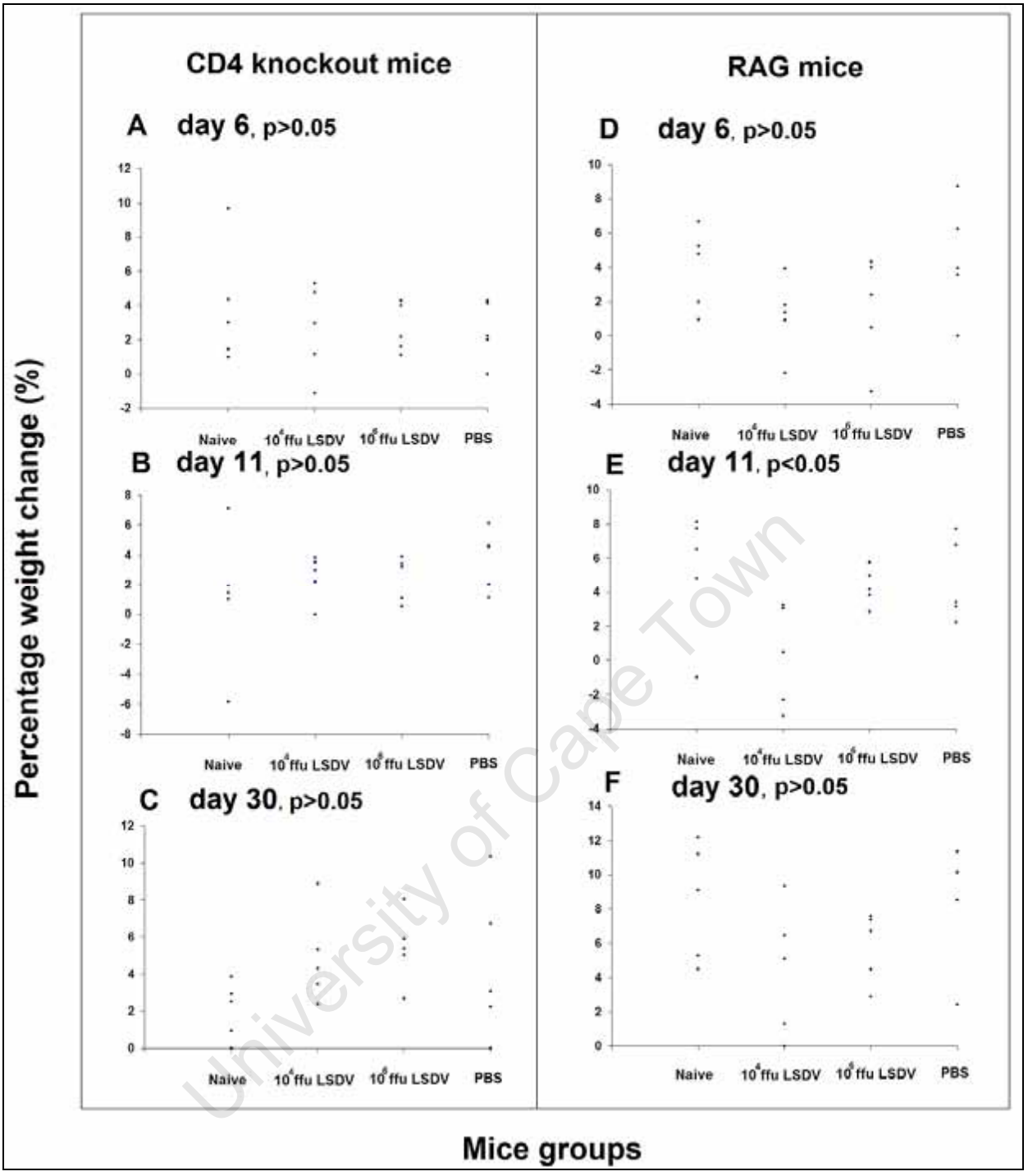


Figure 4.2 Percentage weight change of CD4 knockout mice (A-C) and RAG mice (D-F) in response to vaccination. Mice were vaccinated as indicated. On day 6 (A and D), day 11 (B and E) and day 30 (C and F) the difference in mouse weight from that on day 0 was calculated as a percentage of weight on day 0 and plotted as percentage weight change. A positive value indicates a gain in weight and a negative value indicates a loss in weight on a particular date. A Statitical difference in the percentage weight change for each group was determined using the one-way ANOVA test with 95% confidence interval.

4.3 Cellular Immune Responses to a DNA Prime and Poxvirus Boost Vaccination

Immune responses to rLSDV-Grttn were evaluated in BALB/C mice given either as a single vaccination or as a booster vaccination after a primary vaccination with pVRCgrttnC, a DNA vaccine that encodes the identical antigen Grttn. The immunogenicity of rLSDV-Grttn was compared to another poxvirus vaccine, rMVA-Grttn, a recombinant MVA vaccine that expresses Grttn, controlled by the same VV mH5 promoter. Nine groups of mice were vaccinated as indicated in Chapter 2, Table 2.3, and as summarised in the vaccination and sacrifice time line (Figure 4.3).

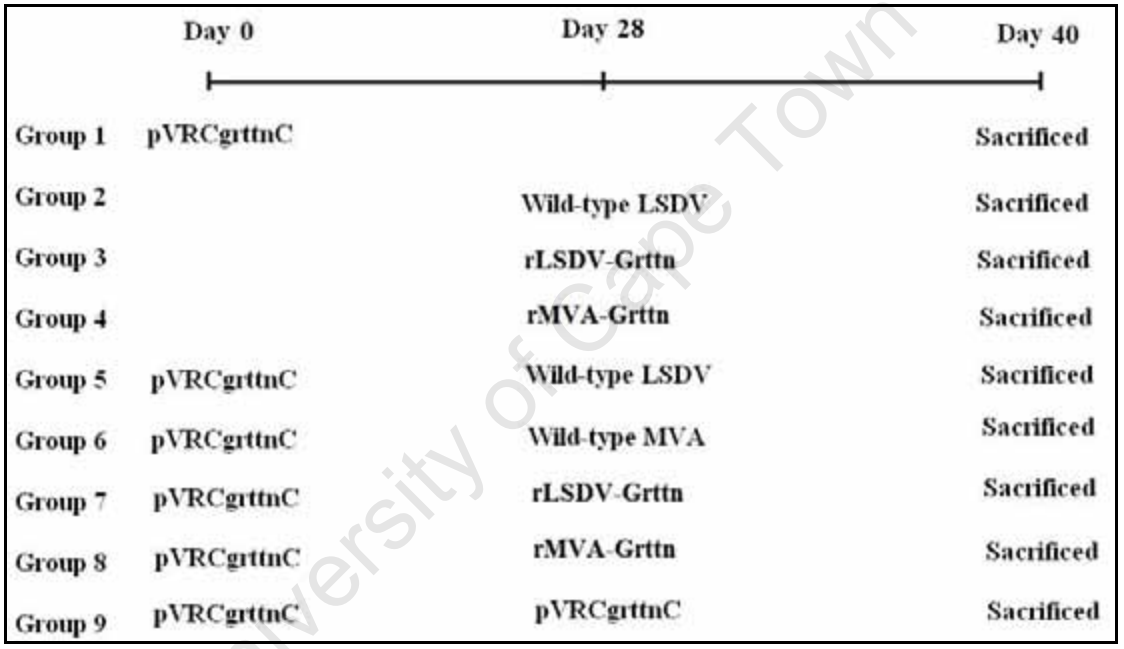


Figure 4.3 BALB/c mouse inoculation and sacrifice schedule to investigate immunogenicity of rLSDV-Grttn alone and when used as a booster vaccine after a prime with pVRCgrttnC (DNA vaccine).

Cellular immune responses were evaluated using IFN-γ and IL-2 ELISPOT assays to enumerate the frequencies of Gag and RT specific IFN-γ or IL-2 secreting CD8+ and CD4+ T cells in the spleen (Figure 4.4A and 4.4B respectively). Considerable background responses in the absence of peptides were detected in both ELISPOT assays (100-300 SFU/10^6 splenocytes) with splenocytes from mice vaccinated with LSDV, (Figure 4.4A and 4.4B). This was seen after a single vaccination or after a boost vaccination with LSDV (wtLSDV or rLSDV-Grttn). Minimal background responses were detected in these assays for vaccination regimens without wtLSDV or rLSDV-Grttn. Responses to an irrelevant peptide were similar to those in the absence of peptide for all vaccination regimens (Figure 4.4A and 4.4B). The magnitude of net positive

responses to individual peptides deduced after background responses were taken into consideration for all vaccination regimens are shown in Figure 4.4C and 4.4D. Cumulative positive responses to the individual HIV peptides for each vaccination regimen is shown in Figure 4.5.

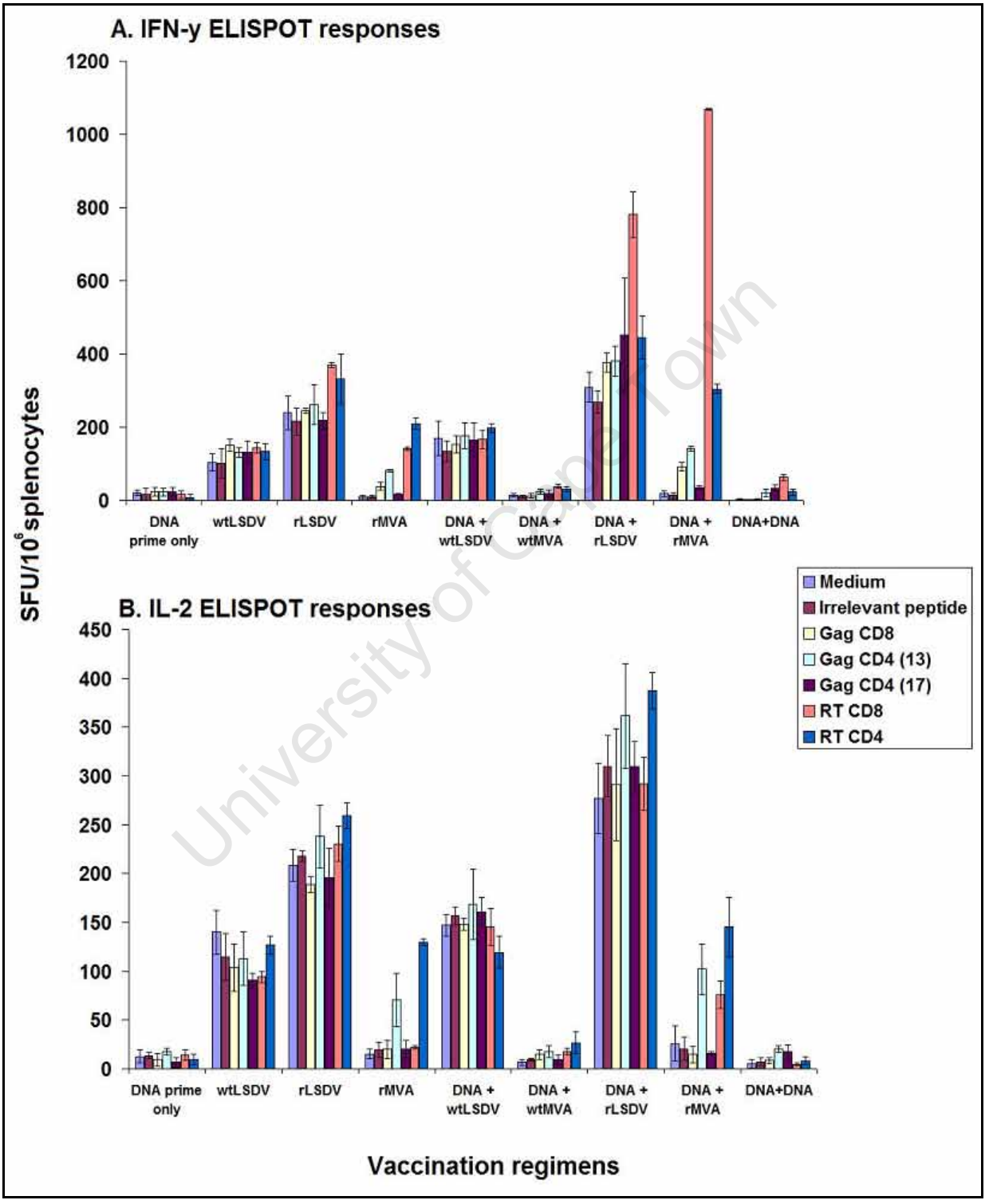


Figure 4.4 Continued to the next page

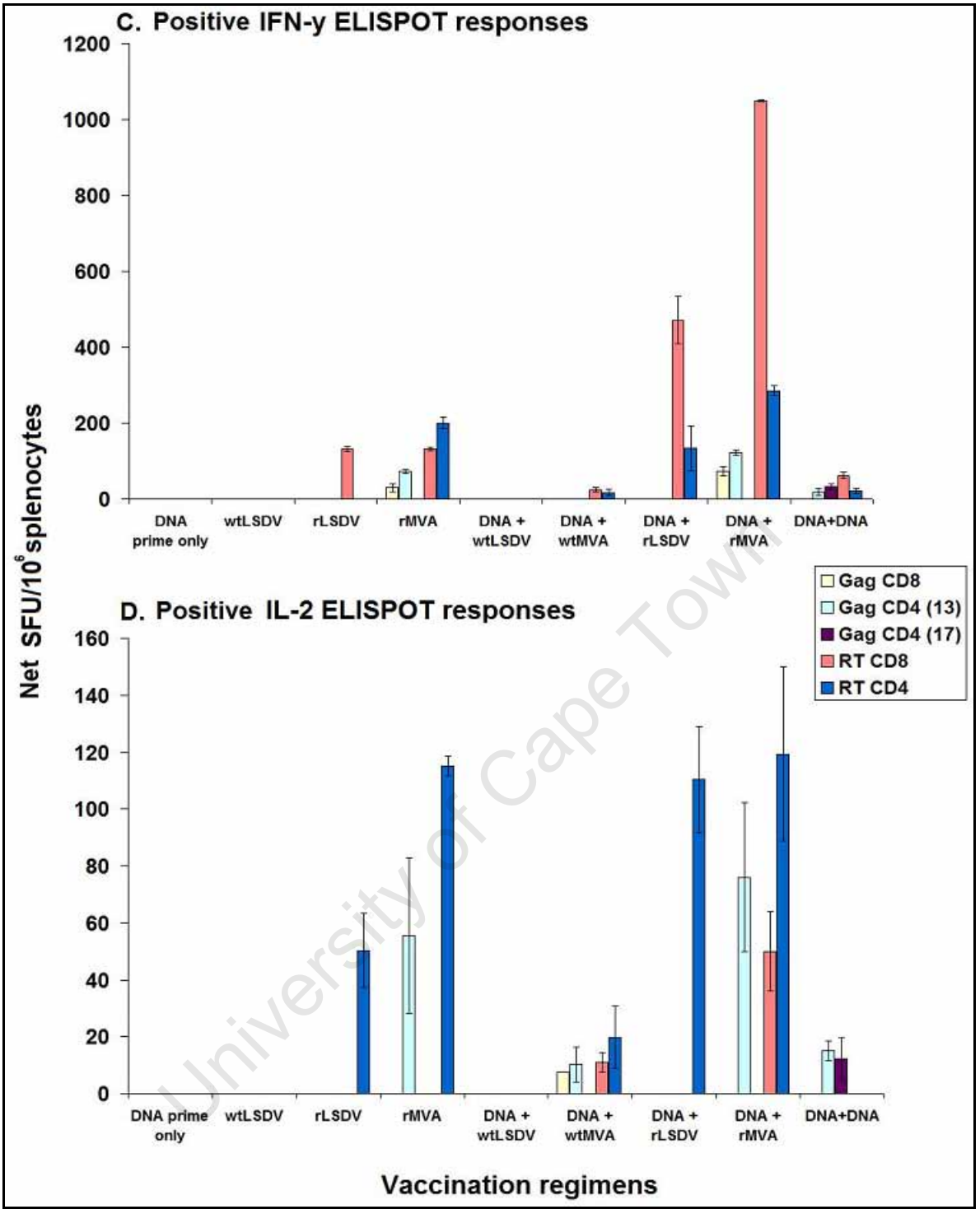


Figure 4.4 IFN-γ and IL-2 ELISPOT analysis of HIV-1 Gag and RT-specific CD8+ and CD4+ T cell responses induced in the spleen by the indicated vaccination regimens and described in Figure 4.3. At sacrifice splenocytes were pooled from 5 mice per group then incubated in the absence of peptide (medium), stimulated with an irrelevant peptide or stimulated with HIV-1 Gag and RT CD8+ and CD4+ T cell peptides in A) IFN-γ ELISPOT and B) IL-2 ELISPOT assays. Bars are the mean number of spots of triplicate reactions in medium only (background) or to an individual peptide for 10^6 splenocytes with indicated standard deviation (SD) of the mean. For each vaccination regimen positive responses to the HIV peptides were considered to be those greater than the background response (response in peptide free medium) plus two times the standard deviation (SD) of this response. C) The magnitude of positive IFN-γ ELISPOT responses and D) the positive IL-2 responses for each vaccination regimen after the background response has been subtracted. The bars indicate the average net SFU ±SD from triplicate wells for 10^6 splenocytes.

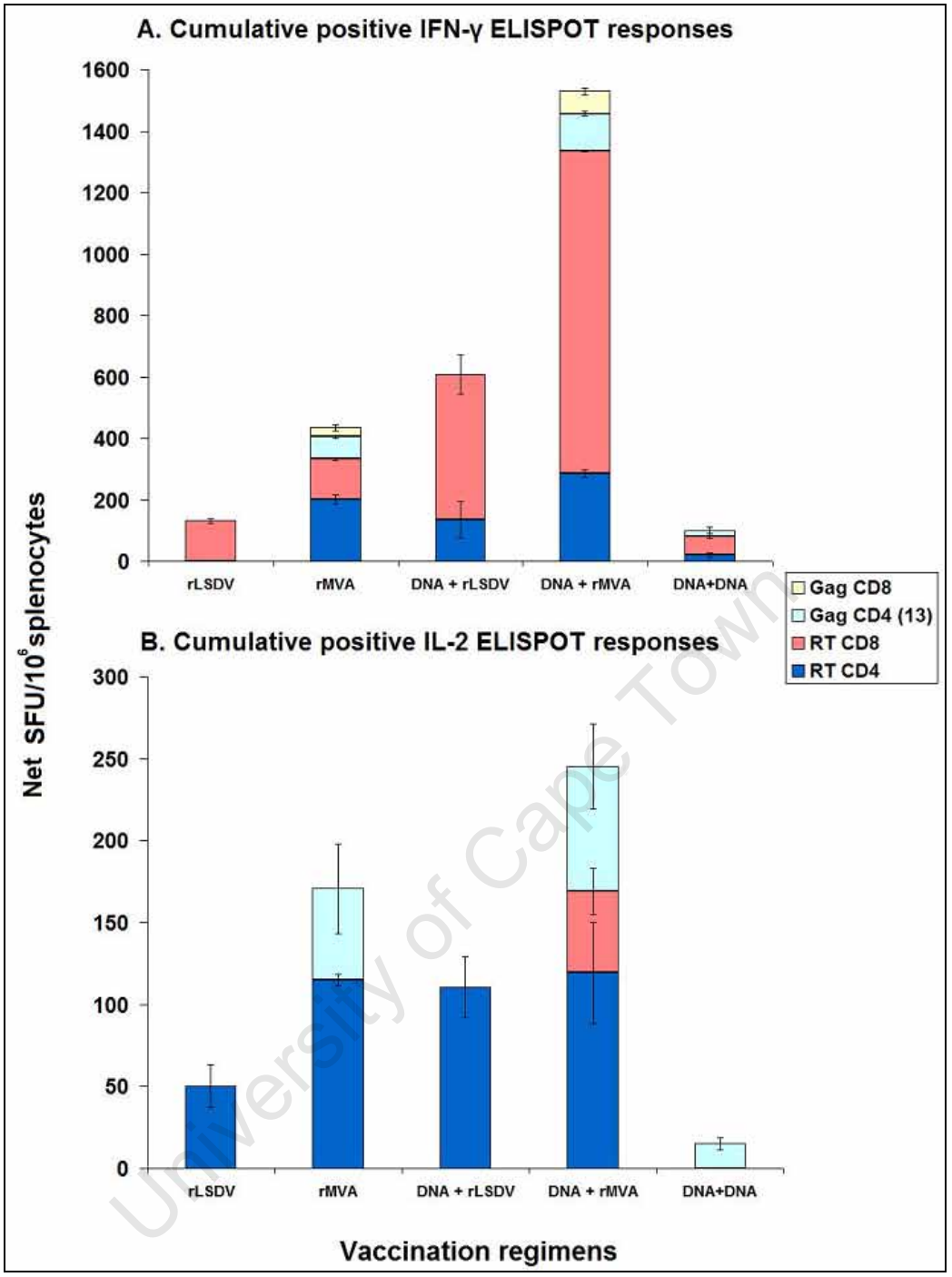


Figure 4.5 Cumulative proportions of positive IFN-γ (A) and IL-2 (B) ELISPOT responses induced by the indicated vaccination regimens and described in Figure 4.3. The individual sections of each bar indicate the average net SFU ±SD from triplicate wells for 10^6 splenocytes to a individual CD8+ or CD4+ peptide in the IFN-γ ELISPOT assay as shown in Figure 4.4 (C and D).

The data from these ELISPOT assays as displayed in Figure 4.4C and 4.4D, indicate wtLSDV did not induce Gag-or RT-specific IFN-γ or IL-2 responses. Vaccination with rLSDV-Grttn induced only a low frequency of RT-specific CD8+ T cells producing IFN-γ (131 Net SFU/10^6 splenocytes) (Figure 4.4C). This is in contrast to a vaccination with rMVA-Grttn which induced responses to Gag and RT CD8+ and CD4+ T cells with a cumulative frequency in the IFN-γ

ELISPOT assay of 435 net SFU/10^6 splenocytes. The ability of these recombinant poxviruses to boost a response induced by a primary pVRCgrttnC DNA vaccination was compared. No Gag or RT responses were detected after the DNA prime (Figure 4.4). However the prime with pVRCgrttnC must have been below the detection limit of the assay as a prime with pVRCgrttnC and boost with rLSDV-Grttn induced a cumulative frequency of RT-specific CD8+ and CD4+ T cells producing IFN-γ of 607 net SFU/10^6 splenocytes of which 78% were from responding RT-specific CD8+ T cells (Figure 4.5A). This cumulative response is 4.6 fold higher than the magnitude of the IFN-γ RT- specific CD8+ T cell response of mice vaccinated with just rLSDV-Grttn. In contrast, mice primed with pVRCgrttnC and boosted with rMVA-Grttn developed responses to Gag and RT CD8+ and CD4+ T cell peptides with a cumulative frequency of 1529 net SFU/10^6 splenocytes (Figure 4.5A). Gag- and RT-specific CD8+ T cells contributed 73% to this cumulative response which was 3.5 fold above that of the response to just rMVA-Grttn. Such boosting of a DNA prime was not detected when the booster vaccines were wtLSDV or wtMVA. These DNA prime-poxvirus boost vaccinations regimens were more potent than a prime and boost with pVRCgrttnC (Figure 4.5A) which induced a cumulative magnitude to Gag and RT CD8+ and CD4+ T peptides of 82 net SFU/10^6 splenocytes in the IFN-γ ELISPOT assay.

Vaccination with rLSDV-Grttn induced RT-specific CD4+ T cells that produced IL-2 (50 net SFU/10^6 splenocytes). In contrast vaccination with rMVA-Grttn induced both Gag CD4+ and RT CD4+ T cells that produced IL-2 (Figure 4.5A). A cumulative frequency of 171 net SFU/10^6 splenocytes was measured. No IL-2 producing cells could be detected in response to a prime with pVRCgrttnC. However when mice were primed with pVRCgrttnC then boosted rLSDV-Grttn, a 2 fold increase in IL-2 producing RT CD4+ T cells over that of rLSDV-Grttn only was measured (Figure 4.5A). A boost of mice primed with pVRCgrttnC with rMVA-Grttn did not change the frequency of RT CD4+ T cells producing IL-2 but increased the frequency of Gag CD4 T cells producing IL-2 by a factor of 2 and induced RT CD8+ T cells producing IL-2 in the ELISPOT assay over that of rMVA-Grttn only (Figure 4.5A). The double vaccination with DNA induced a low frequency of Gag CD4+ T cells producing IL-2 (15 net SFU/10^6 splenocytes) (Figure 4.5A).

4.4 Cellular Immune Responses to Poxvirus Prime and Boost Vaccination Regimens

Immune responses of BALB/c mice to rLSDV-Grttn were evaluated after either homologous or heterologous poxvirus prime/boost regimens with rMVA-Grttn. Nine groups of BALB/C mice were vaccinated as indicated in (Table 2.4A) and as summarised in the vaccination and sacrifice time line (Figure 4.6)

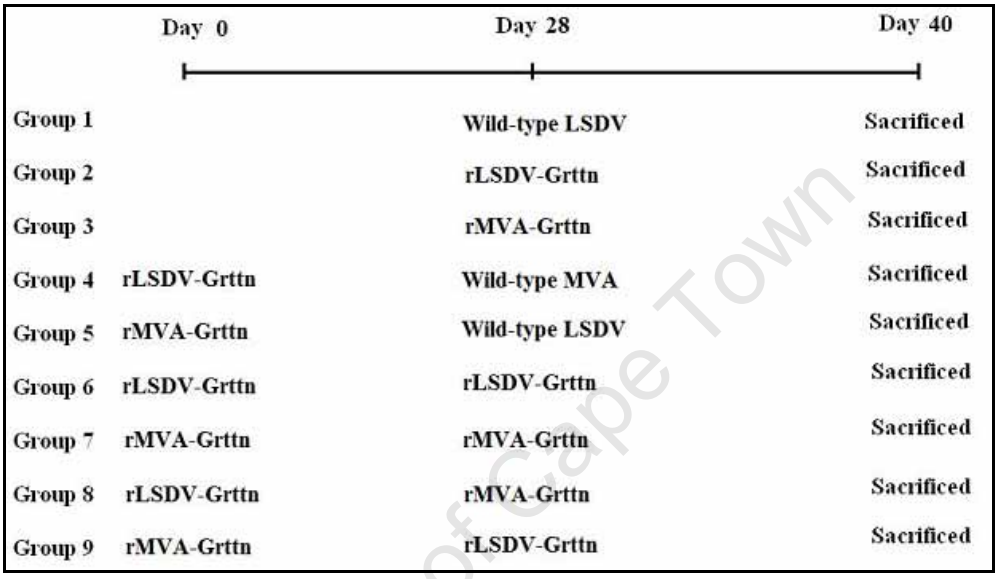


Figure 4.6 BALB/c mouse inoculation and sacrifice schedules to investigate immunogenicity of rLSDV-Grttn alone and in homologous and heterologous and prime-boost combinations with rMVA-Grttn.

Cellular immune responses were evaluated using IFN-γ and IL-2 ELISPOT assays to enumerate the frequencies of Gag and RT specific IFN-γ or IL-2 secreting CD8+ and CD4+ T cells in the spleen (Figure 4.7A and 4.7B respectively). As we found in our previous experiments considerable background responses in the absence of peptides were detected in both ELISPOT assays of 80-370 SFU/10^6 splenocytes (Figure 4.7A and 4.7B) for splenocytes harvested from mice vaccinated with LSDV. This occurred for both recombinant and wtLSDV, and after both a single vaccination and when LSDV was used in prime/boost vaccination regimens. Minimal background responses were detected in these assays for vaccination regimens without wtLSDV or rLSDV-Grttn. Responses to an irrelevant peptide were similar to those in the absence of peptide for all vaccination regimens (Figure 4.7A and 4.7B). The magnitude of net positive responses to individual peptides deduced after background responses were taken into consideration for all vaccination regimens are shown in Figure 4.7C and 4.7D. Cumulative positive responses to the individual HIV peptides for each vaccination regimen is shown in

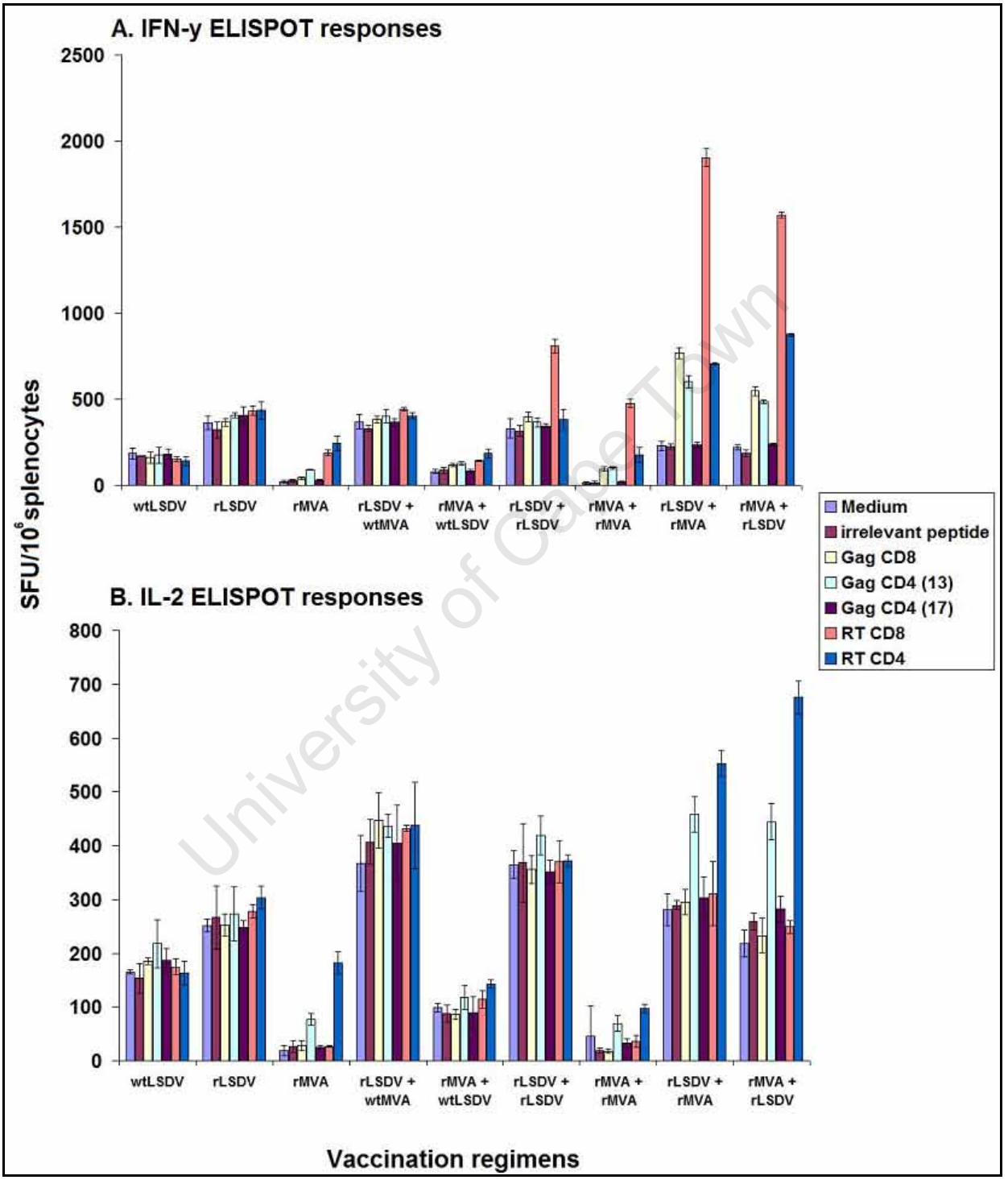
Figure 4.8.

In this set of experiments no response in the IFN-γ ELISPOT assay was detected for mice vaccinated with rLSDV-Grttn alone. A single vaccination with rMVA-Grttn induced Gag and RT-specific CD4+ and CD8+ T cell responses with a cumulative magnitude of 475 net SFU/10^6 splenocytes. When rLSDV-Grttn was used in a homologous prime/boost vaccination regimen, only RT-specific CD8+ T cells with a magnitude of 477 net SFU/10^6 splenocytes were detected in the IFN-γ ELISPOT assay (Figure 4.8A). The homologous prime/boost vaccination regimen with rMVA-Grttn induced Gag and RT-specific CD4+ and CD8+ T cell responses with a cumulative magnitude of 795 net SFU/10^6 splenocytes (Figure 4.8A), with 68% of the response due to responding CD8+ T cells. This cumulative response is 1.8 fold higher than the magnitude of cumulative Gag and RT-specific response induced by rMVA-Grttn alone (475 net SFU/10^6 splenocytes).

The heterologous prime/boost vaccinations were more potent regimens compared to the homologous vaccination regimens. After priming with rMVA-Grttn and boosting with rLSDV-Grttn or priming with rMVA-Grttn and boosting with rLSDV-Grttn, high frequencies of IFN-γ secreting Gag and RT specific CD8+ and CD4+ T cells were induced (Figure 4.8A). Cumulative frequencies of Gag and RT-specific CD4+ and CD8+ T cell producing IFN-γ of 3066 net SFU/10^6 splenocytes was induced by a rLSDV-Grttn prime/ rMVA-Grttn boost vaccination regimen, with 72% of the response contributed by the CD8+ T cells. This cumulative response is 6.5 fold higher than the magnitude of cumulative frequency induced by rMVA-Grttn alone. The reverse vaccination regime induced a cumulative frequency of Gag and RT-specific, CD4+ and CD8+ T cell producing IFN-γ of 2592 net SFU/10^6 splenocytes of, which 65% was due to responding CD8+ T cells. This cumulative response is 5.5 fold higher than the magnitude of cumulative frequency induced by rMVA-Grttn alone (Figure 4.8A).

IL-2 producing cells were detected in the IL-2 ELISPOT assay. A single vaccination of rLSDV-Grttn induced a low frequency of RT-specific CD4+ T cells producing IL-2 of 52 net SFU/10^6 splenocytes. Gag- and RT-specific CD4+ T cells producing IL-2 with a cumulative frequency of 221 net SFU/10^6 splenocytes were induced by a single vaccination of rMVA-Grttn. However, no detectable positive IL-2 ELISPOT responses were measure in mice vaccinated with a homologous prime/boost vaccination regimen for either rLSDV-Grttn or rMVA-Grttn. In contrast, a prime with rLSDV-Grttn and boost with rMVA-Grttn resulted in a boost of both Gag and RT-specific CD4+ T-cells to a cumulative magnitude of IL-2 producing cells of 450 net SFU/10^6 splenocytes. The cumulative magnitude of IL-2 producing cells reached 750 net

SFU/10^6 splenocytes when mice were primed with rMVA-Grttn then boosted with rLSDV-Grttn (Figure 4.8B). The rMVA-Grttn prime/ rLSDV-Grttn boost regimen induced a low IL-2 ELISPOT response specific for Gag CD4 (17) peptide (64 net SFU/10^6 splenocytes) (Figure 4.7D), which was absent in the reverse vaccination regimen (Figure 4.7D).



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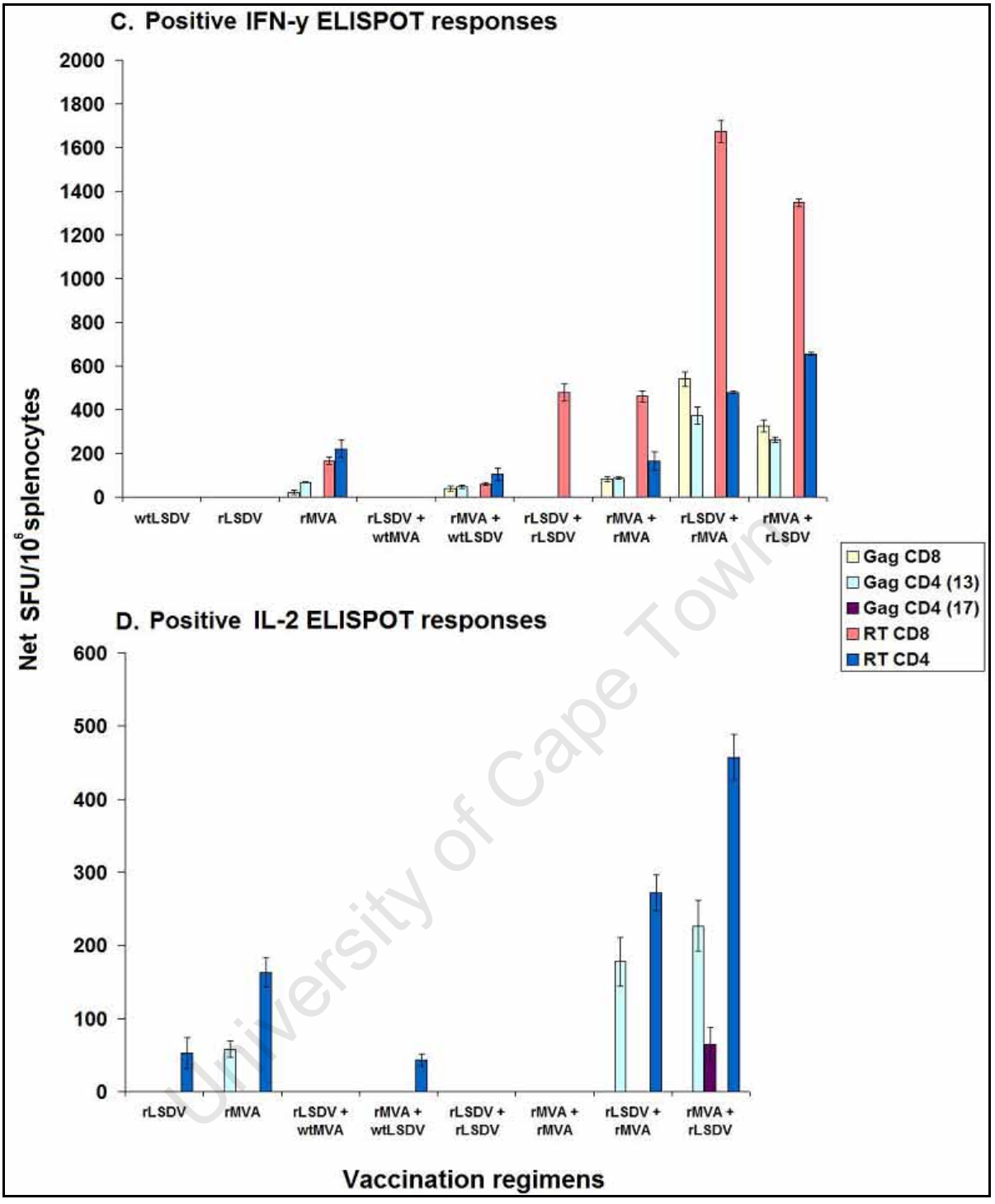


Figure 4.7 IFN-γ and IL-2 ELISPOT analysis of HIV-1 Gag and RT-specific CD8+ and CD4+ T cell responses induced in the spleen by the indicated vaccination regimens and described in Figure 4.6. At sacrifice splenocytes were pooled from 5 mice per group then incubated in the absence of peptide (medium), stimulated with an irrelevant peptide or stimulated with HIV-1 Gag and RT CD8+ and CD4+ T cell peptides in A) IFN-γ ELISPOT and B) IL-2 ELISPOT assays. Bars are the mean number of spots of triplicate reactions in medium only (background) or to an individual peptide for 10^6 splenocytes with indicated standard deviation (SD) of the mean. For each vaccination regimen positive responses to the HIV peptides were considered to be those greater than the background response (response in peptide free medium) plus two times the standard deviation (SD) of this response. C) The magnitude of positive IFN-γ ELISPOT responses and D) the positive IL-2 responses for each vaccination regimen after the background response has been subtracted. The bars indicate the average net SFU ±SD from triplicate wells for 10^6 splenocytes. The magnitude of positive IL-2 ELISPOT responses of each group after subtracting the background responses, with the bars indicating the average net SFU ±SD from the triplicate wells for 10^6 splenocytes.

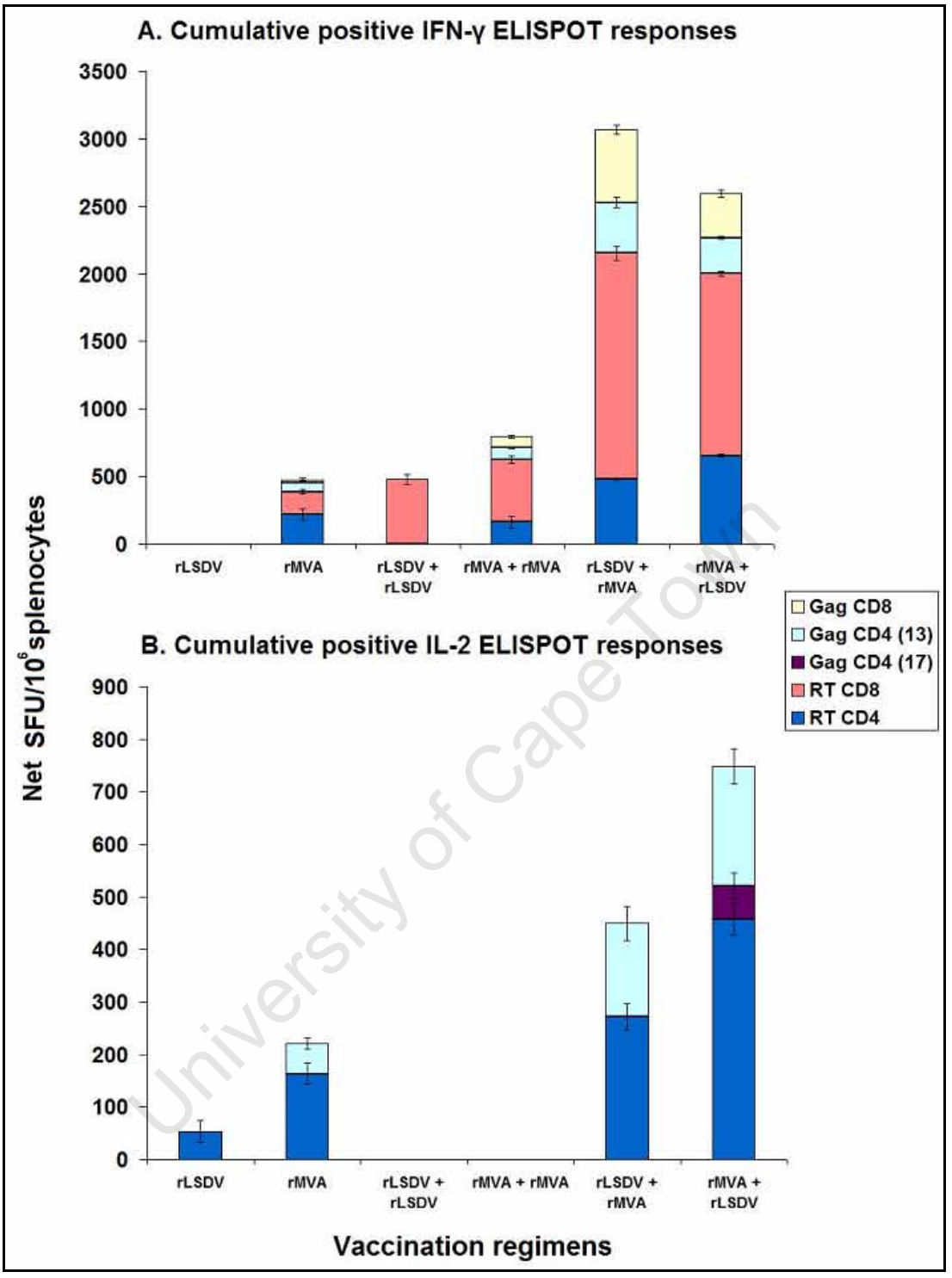


Figure 4.8 Cumulative proportions of positive IFN-γ (A) and IL-2 (B) ELISPOT responses induced by the indicated vaccination regimens and described in Figure 4.6. The individual sections of each bar are the average net SFU ±SD from triplicate wells for 10^6 splenocytes to an individual CD8+ or CD4+ peptide in the IFN-γ ELISPOT assay as shown in Figure 4.7 (C and D).

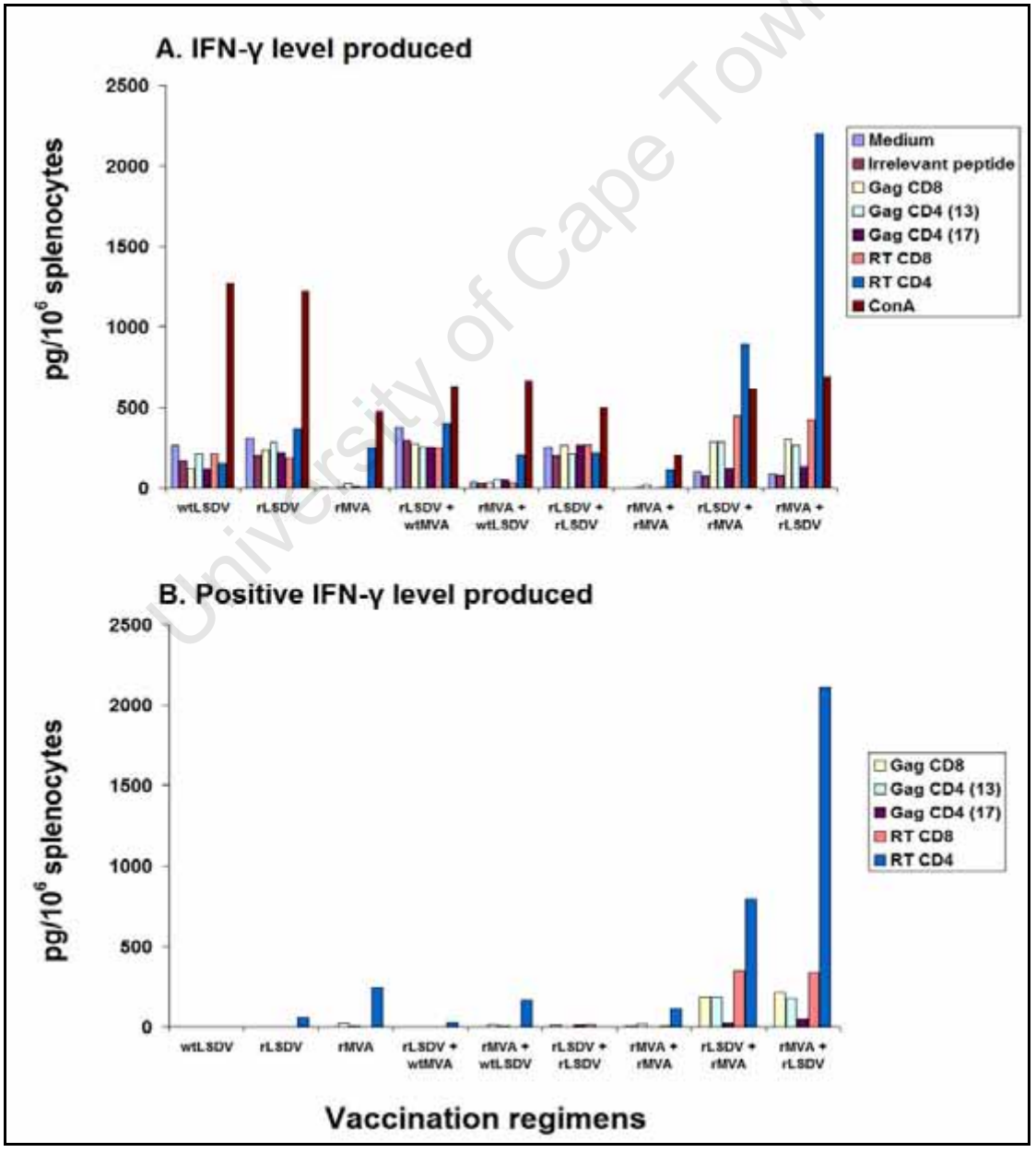
4.5 Gag- and RT- specific IFN-γ Levels Induced by Poxvirus Prime/ Boost Vaccination Regimens

The amount of IFN-γ secreted by the Gag- and RT-specific CD4+ and CD8+ T cells during culture with the HIV peptides was also quantified (Figure 4.9). Splenocytes harvested from mice vaccinated according to the regimens indicated in Figure 4.6 were incubated for 48h with the Gag- and RT- specific CD8+ and CD4+ T cell peptides and the level of IFN-γ released into the culture medium was quantified using a Cytometric Bead Array assay (BD Pharmingen) and flow cytometric analysis.

Considerable background IFN-γ of 86-376 pg/10^6 splenocytes was released into the medium when splenocytes were incubated in the absence of peptides for splenocytes harvested from mice vaccinated with LSDV, both recombinant and wild-type, and after both a single vaccination and when LSDV was used in prime/boost vaccination regimens (Figure 4.9A). Minimal background IFN-γ was released by splenocytes from mice vaccinated with the other vaccine regimens (Figure 4.9A). IFN-γ released by splenocytes to an irrelevant peptide was similar to that in the absence of peptide for all vaccination regimens (Figure 4.9A). The magnitude of the net positive IFN-γ level produced by the splenocytes during culture with individual CD8+ and CD4+ T cell peptides deduced after background responses were taken into consideration are shown in Figure 4.9B for all vaccination regimens. The cumulative positive IFN-γ level produced to the HIV peptides for the individual vaccination regimens is shown in Figure 4.9C.

A low level of IFN-γ was released from RT-specific CD4+ T cells (57 pg/10^6 splenocytes) in response to a rLSDV-Grttn vaccination even though we could not detect these cells in an IFN-γ ELISPOT assay (Figure 4.7C). After a single vaccination with rMVA-Grttn, RT-specific CD4+ T cells producing IFN-γ (267 pg/10^6 splenocytes) were detected (Figure 4.9C). WtLSDV and WtMVA did not boost these HIV-1 specific IFN-γ levels. The level of IFN- γ produced by splenocytes from homologous prime/boost vaccinations with rLSDV-Grttn or rMVA-Grttn was similar to that of a single vaccination with rLSDV-Grttn or rMVA-Grttn. In contrast, splenocytes from heterologous prime/boost vaccination regimens produced high levels of IFN-γ during culture with Gag- and RT-specific peptides (Figure 4.9C). Splenocytes from mice primed with rLSDV-Grttn and boosted with rMVA-Grttn produced a cumulative positive IFN-γ level of 1540 pg/10^6 splenocytes, with 52% of the IFN-γ produced by RT-specific CD4+ T cells (Figure 4.9C). Splenocytes from mice vaccinated with the reverse vaccination regimen produced a cumulative

positive IFN-γ level of 2889 pg/10^6 splenocytes. 73% of this total IFN-γ was produced by RT-specific CD4+ T cells (Figure 4.9C). Thus the difference in the level of IFN-γ produced by the two heterologous prime/boost regimens was mainly attributed to a higher level of IFN-γ being produced by the RT-specific CD4+ T cells. This data leads to the suggestion that a prime with rMVA-Grttn and boost with rLSDV-Grttn is more potent than the reverse inoculation regimen (Figure 4.9C). However, the data from the IFN-γ ELISPOT assay indicates only a small difference in the frequencies of RT-specific CD4+ T cells (Figure 4.8A) for these two heterologous prime boost vaccination regimens (Figure 4.8A). Thus the RT-specific CD4+ T cells induced by the rMVA-Grttn prime/rLSDV-Grttn boost have a higher capacity to produce IFN-γ per cell than the RT-specific CD4+ T cells induced by the rLSDV-Grttn prime/rMVA-Grttn boost.



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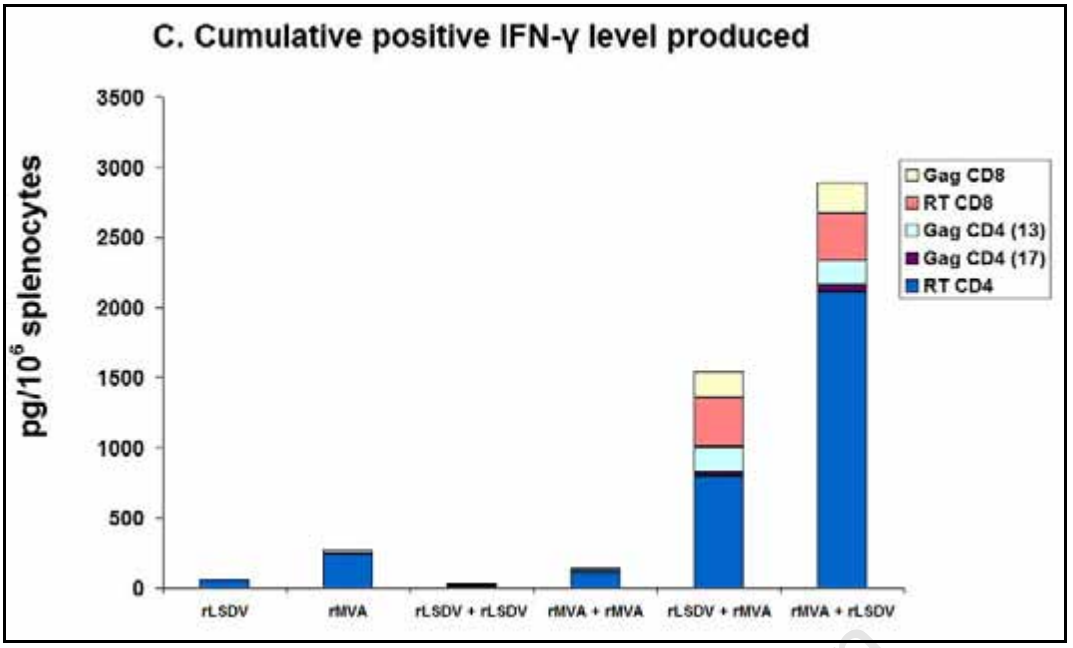

Figure 4.9 IFN-γ produced by Gag- and RT-specific CD8+ and CD4+ T cells during culture. Splenocytes was harvested from mice vaccinated with the indicated regimens and described in Figure 4.6, and pooled from 5 mice per group. They were then cultured in the absence of peptide (medium), an irrelevant peptide or with the individual Gag- and RT-specific CD8+ and CD4+ T cell peptides for 48 h. Culture supernatants were collected and the level of IFN-γ released into the medium (pg /10^6 splenocytes) was measured using a Cytokine Bead Array assay and flow cytometric analysis. A) IFN-γ level in the splenocyte culture medium. The individual bars represent the magnitude of the IFN-γ level in the medium of splenocyte of vaccinated mice, cultured with individual peptides B) Magnitude of positive IFN-γ produced in the culture medium. The level of IFN-γ in the culture medium was considered positive when the level is higher than the IFN-γ level in medium of splenocytes cultured in the absence of peptide. The individual bars represent the magnitude of the IFN-γ level in the medium of splenocyte of vaccinated mice, cultured with individual peptides, after subtracting the IFN-γ level in medium of splenocytes cultured in the absence of peptide from it. C) Cumulative proportions of positive Gag- and RT-specific CD4+ and CD8+ T cell IFN-γ levels. Individual bars represent the cumulative positive IFN-γ levels in the culture medium of splenocytes of vaccinated mice. The contributions due to different peptide stimulations are colour coded as indicated.

4.6 Phenotype of Pentamer-positive HIV-1-specific CD8+ T cells Induced by rLSDV-Grttn and rMVA-Grttn Vaccination Regimens

Total Gag- and RT- specific CD8+ T cells induced in the spleens of mice by the rLSDV-Grttn prime/ rMVA-Grttn boost regimen and the reverse prime/ boost vaccination regimen were enumerated. CD8+ T cells in the spleens of vaccinated mice that bound either a H-$2D^k$ MHC class I pentamer folded with the Gag CD8 peptide (AMQMLKDTI) or the RT CD8 peptide (VYYDPSKDLIA) were analysed as a percentage of total CD8+ T cells by flow cytometry. Expression of CD44 on these pentamer+ CD8+ T cells was also evaluated. For these studies mice were vaccinated as indicated in Figure 4.10.

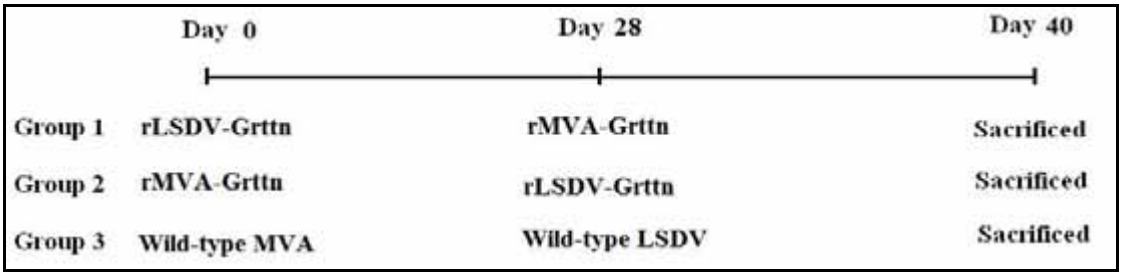

Figure 4.10 BALB/c mouse inoculation and sacrifice schedules to investigate phenotype of HIV-specific CD8+ T cells induced by the vaccination regimens and investigate the effect of foetal calf serum on background responses in the IFN-γ ELISPOT assay.

Flow cytometric analysis indicated that both vaccination regimens induced high frequencies of HIV-specific CD8+ T cells while vaccination with wild-type virus induced low unspecific Gag and RT pentameric complex binding of 0.5% and 0.7% of CD8+ T cells respectively (Figure 4.11A). The sum of Gag- and RT-specific CD8+ T cells was 6.9% of the total CD8+ T cells for rLSDV-Grttn prime/ rMVA-Grttn boost vaccination regimen. This frequency was 4.6% of the gated CD8+ T cells with the reverse vaccination regimen. For both regimens approximately 98% of these HIV-specific CD8+ T cells expressed CD44 confirming these T cells to be antigen experienced.

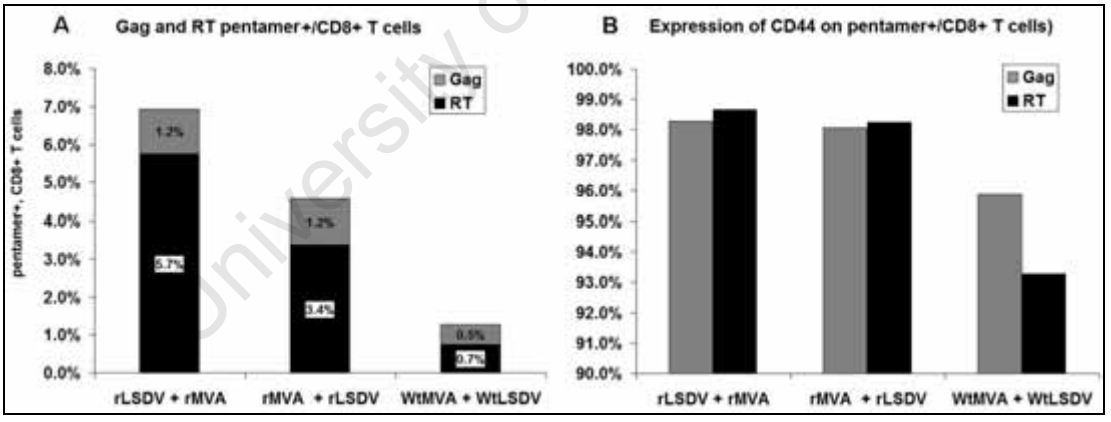

Figure 4.11.Expression of CD44 on Gag- and RT-specific CD8+ T cells. Mice were vaccinated as indicated and according to the schedule described in Figure 4.10. At sacrifice splenocytes were pooled from 5 mice per group. Vaccine elicited Gag- and RT- specific CD8+ T cells in the splenocyte population were detected by binding of a H-2D^{k} MHC class I pentamer folded with either the Gag CD8 peptide (AMQMLKDTI) or the RT CD8 peptide (VYYDPSKDLIA) to CD8+ T cells using flow cytometry. A) Data presented as the percentage of gated CD8+ T cells that bound the pentamer B) Percentage of pentamer+/CD8+ T cells that expressed CD44 in the pentamer+/CD8+ T cell gate.

4.7 Expression of Degranulation Marker CD107a/b on HIV-1 specific CD8+ T cells

The expression of CD107a/b on the HIV-1 specific CD8+ T cells induced by the rLSDV-Grttn prime/ rMVA-Grttn boost regimen and the reverse vaccination regimen in BALB/C mice was evaluated as a further functional characteristic of these CD8+ T cells. The splenocytes from each group of mice vaccinated as indicated in Figure 4.10 were stimulated with either Gag or RT CD8 peptides or left unstimulated. Flow cytometry was used to enumerate the Gag- and RT-specific CD8+ T cells that bound the H-2D^k MHC class I pentamer folded with either the Gag or the RT CD8 peptide and then expression of CD107a/b on these pentamer+ CD8+ T cells .

Stimulation of splenocytes with Gag or RT CD8 peptides had no or little effect on the frequency of H-2D^k MHC class I pentamer binding (Figure 4.12) and confirmed the high frequency of HIV-1 specific CD8+ T cells induced by rLSDV-Grttn prime/ rMVA-Grttn boost regimen and the reverse regimen as shown in Figure 4.11A and 4.11B. Also as previously found vaccination with wild-type virus did not induce HIV-1 specific CD8+ T cells (Figure 4.11A). For both vaccination regimens a high percentage (>61%) of these HIV-specific pentamer+ CD8+ T cells were found to express CD107a/b after stimulation (Figure 4.12C and 4.12D). However, a large percentage of the unstimulated HIV-1 specific CD8+ T cells from both regimens were also positive for CD107a/b (57.2%-75.8%) (Figure 4.12C and 4.12D), indicating a large percentage of these HIV-1 specific CD8+ T cells *ex vivo* may express CD107a/b before stimulation. Alternatively, there is non-specific expression of this marker during culture in medium alone. To address this, the experiment should have included the analysis of CD107a/b expression on the splenocytes before the six hour peptide stimulation (time zero control).

4.8 Role of Foetal Bovine Serum on Background Responses in the IFN-γ ELISPOT Assay

Background responses in the IFN-γ and IL-2 ELISPOT assays were abnormally high (Figure 4.4 and 4.7) for splenocytes from mice vaccinated with either wtLSDV or rLSDV-Grttn. There is a possibility that these responses are a result of responses to components of the foetal bovine serum in the culture medium used in the assays. This was investigated by performing the assays in a serum free medium (SFM; CTL TEST^{TM} medium, see Chapter 2 Methods and materials). Splenocytes from mice vaccinated as indicated in Figure 4.10 were used in an IFN-γ ELISPOT assay with either the conventional culture medium (R10) containing foetal bovine serum or SFM.

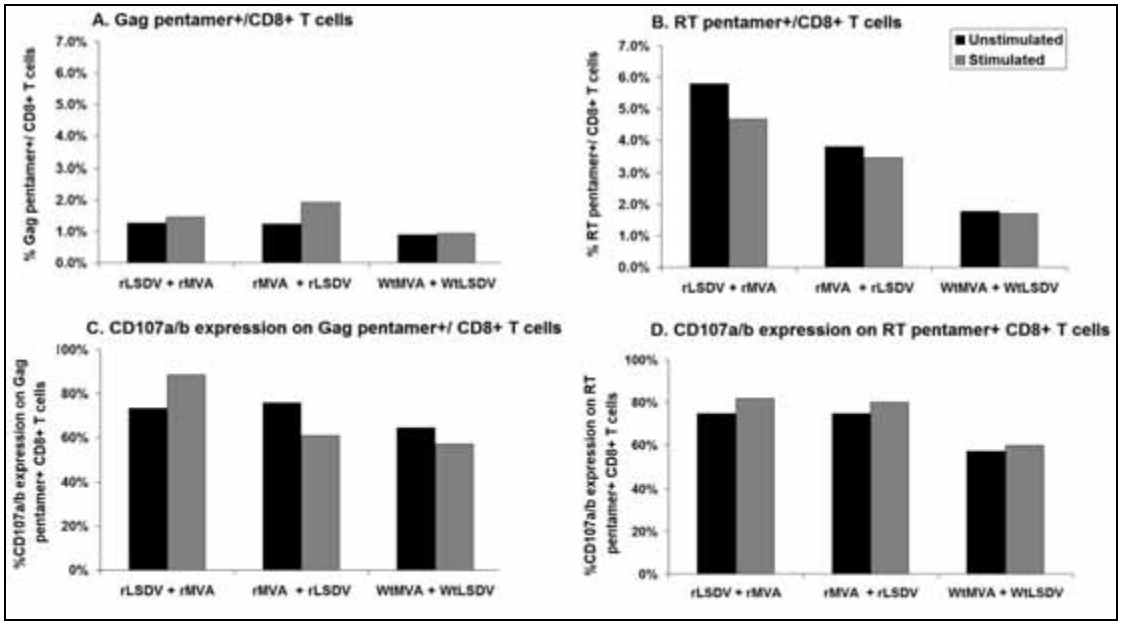

Figure 4.12. Expression of CD107a/b on Gag- and RT-specific CD8+ T cells. Mice were vaccinated as indicated and according to the schedule described in Figure 4.10. At sacrifice splenocytes were pooled from 5 mice per group then stimulated with either Gag or RT CD8 peptides or left unstimulated. A and B: Flow cytometry analysis if Gag- and RT- specific CD8+ T cells binding of H-2D^k MHC class I pentamers folded with either the Gag CD8 peptide (AMQMLKDTI) or the RT CD8 peptide (VYYDPSKDLIA). Data is presented as the percentage of gated CD8+ T cells that bound the pentamer. C and D: Percentage of pentamer+/CD8+ T cells that expressed CD107a/b in the pentamer+/CD8+ T cell gate.

Background responses in the IFN-γ ELISPOT assay for splenocytes in R10 for this set of experiments (Figure 4.13) were 2.4 fold lower (rLSDV-Grttn primed/ rMVA-Grttn regimen) or 5.4 fold lower (rMVA-Grttn primed/ rLSDV-Grttn regimen) than reported in the previous IFN-γ assays (see Figure 4.4 or 4.7). However, these background responses of 41-92 SFU/10^6 splenocytes obtained in this experiment are still considered higher than the background responses for vaccination regimens where LSDV in not administered. In the IFN-γ ELISPOT assays with SFM, the background responses were found to range from 1-9 SFU/10^6 splenocytes which is 10-41 fold reduced from that obtained in the IFN-γ ELISPOT with R10 (Figure 4.13). However the Gag- and RT- specific IFN-γ ELISPOT responses to both poxvirus prime boost regimens for the assay with R10 and SFM were comparable. This may suggest that the background responses in the absence of peptide do not influence specific responses in the presence of peptide.

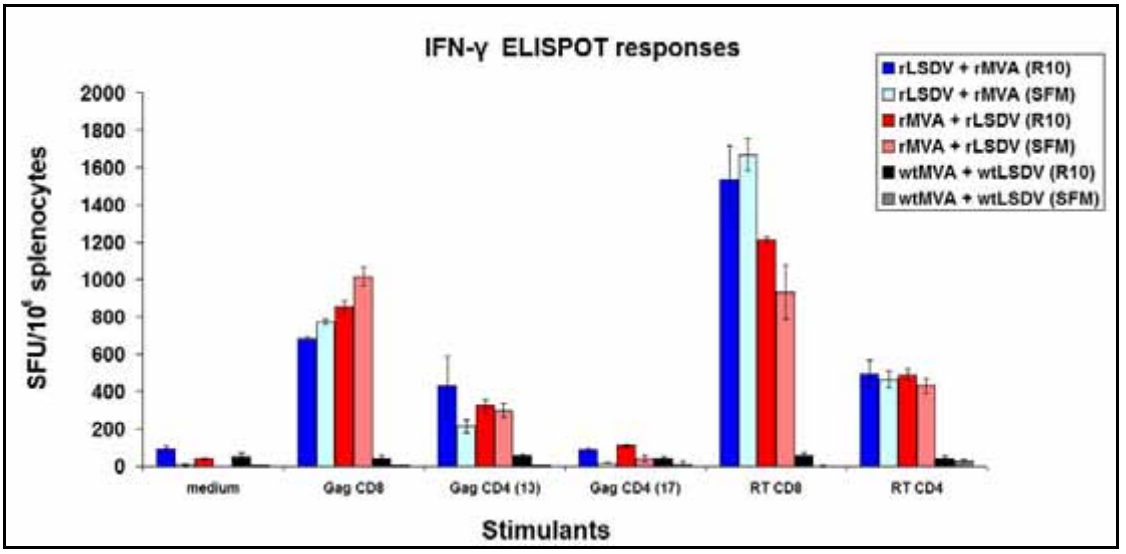

Figure 4.13 Comparison of IFN-γ ELISPOT assays in R10 and SFM. Mice were vaccinated as indicated and according to the schedule described in Figure 4.10. At sacrifice splenocytes were pooled from 5 mice per group then used in an IFN-γ ELISPOT assay with either R10 culture medium or SFM as indicated without peptides (medium) or the indicated HIV-1 Gag and RT CD8+ and CD4+ T cell peptides. Bars are the mean number of spots of triplicate reactions in medium only (background) or to an individual peptide for 10^6 splenocytes with indicated standard deviation (SD) of the mean.

4.9 Comparison of the Frequency of HIV-1 specific CD8+ T Cells Detected in the IFN-γ ELISPOT Assay and by Pentameric H-2D^k Peptide Complex Binding to HIV-specific CD8+ T Cells

The frequencies of Gag- and RT-specific CD8+ T cells detected in the IFN-γ ELISPOT assay performed in SFM (Figure 4.13) were calculated as a percentage of the total CD8+ T cell population in the spleen (Table 4.1). These figures were then compared with the percentage of HIV-specific CD8+ T cells obtained using pentameric H-2D^k peptide complex binding (Table 4.1 and see Figure 4.11). For the rLSDV-Grttn prime/ rMVA-Grttn boost regimen the percentage of Gag- or RT-specific CD8+ T cells detected in the IFN-γ ELISPOT assay was calculated to be 0.57% and 1.23% of the CD8+ T cell population in spleen respectively (Table 4.1). These figures for the reverse regimen were calculated to be 0.73% and 0.68% of the CD8+ T cell population in the spleen (Table 4.1). When this data is compared to the data obtained using pentameric H-2D^k peptide complex binding to enumerate these HIV-specific CD8+ T cells it appears that both regimens may induce more than just HIV-specific IFN-γ producing cells.

Table 4.1 Comparison of frequencies of Gag- and RT- specific CD8+ T cells detected by IFN-γ ELISPOT or pentameric H-2D^k peptide complex binding. Mice were vaccinated as indicated and according to the schedule described in Figure 4.10. At sacrifice splenocytes were pooled from 5 mice per group. CD8+ T cells in the splenocyte population was determined by flow cytometry and presented as percentage gated lymphocytes. Vaccine elicited Gag- and RT- specific CD8+ T cells in the splenocyte population were detected using an IFN-γ ELISPOT assay and data presented as SFU/10^6 splenocytes. Gag- and RT- specific CD8+ T cells in the splenocyte population were also detected using binding of H-2D^k MHC class I pentamers folded with either the Gag CD8 peptide (AMQMLKDTI) or the RT CD8 peptide (VYYDPSKDLIA) to CD8+ T cells and data is presented as the percentage of gated CD8+ T cells that bound the pentamer as measured by flow cytometry.

Vaccination regimen	**HIV specific response**	**% CD8+ T-cells in spleen**	**CD8+ T cells / 10^6 splenocytes**	**ELISPOT SFU/10^6 splenocytes (See Figure 4.13)**	**SFU/10^6 splenocytes expressed as a % of total CD8+ T cells**	**% Pentamer +/ CD8+ T cells (See Figure 4.12A)**
rLSDV+rMVA	Gag	13.5	135000	765	765/135000 = 0.57%	1.19%
rLSDV+rMVA	RT	13.5	135000	1663	1663/135000 = 1.23%	5.74%
rMVA+rLSDV	Gag	13.8	138000	1014	1014/138000 = 0.73%	1.20%
rMVA+rLSDV	RT	13.8	138000	933	933/138000 = 0.68%	3.37%

4.10 Antibody Responses Induced by the Vaccination Regimens

The HIV-1 specific antibody responses induced by the various single and prime/boost vaccination regimens as indicated in Figure 4.3 and 4.6 were evaluated using New LAV Blot I Assay. BALB/C mice were pre-bled to determine background antibodies then at the end of the experiment before spleens were harvested.

Figure 4.14 shows the sera incubated test strips of single and DNA prime /poxvirus boost regimens. DNA prime/ rMVA-Grttn boost regimen elicited weak antibody response for Gag (p25). No detectable HIV-1 specific antibody was induced by the other vaccination regimens. Nonspecific antibodies binding to the test strip (position slightly above p68) were induced by vaccination regimens LSDV (wild-type and recombinant).

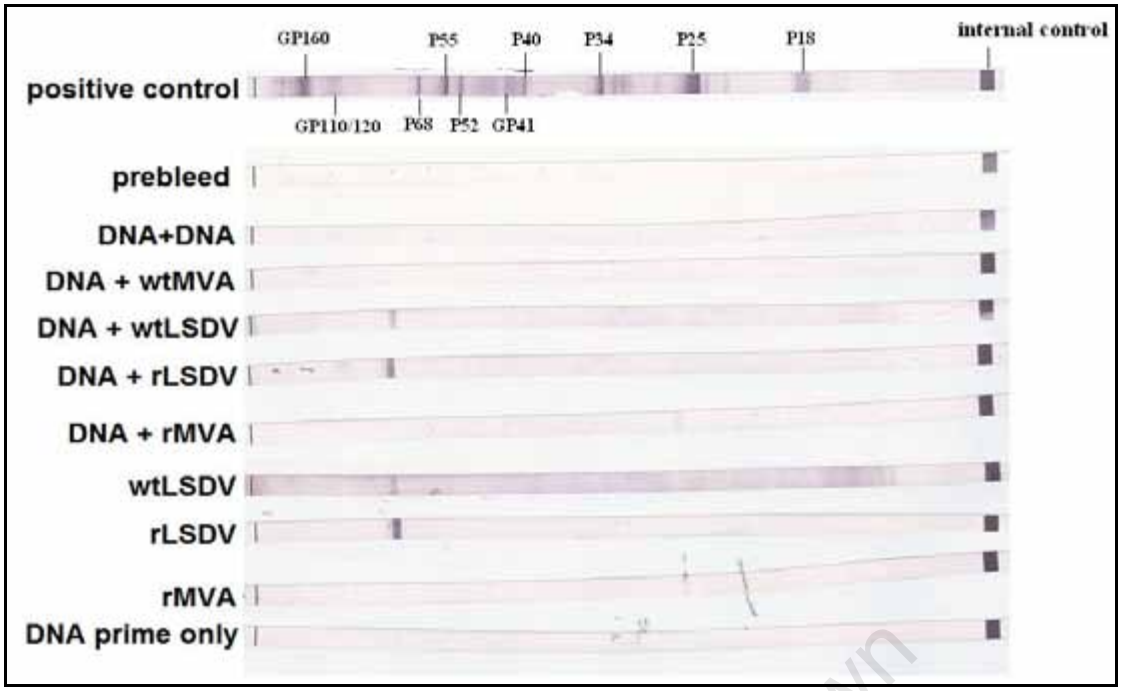

Figure 4.14 HIV-1 specific antibody in sera of mice vaccinated with single vaccines or DNA prime/boost vaccination regimens indicated in the Figure 4.3. On Day 40, sera were pooled from five mice per group. Sera obtained from the prebleed prior vaccination was included as negative control. The presence of HIV-1 antibody in these sera was detected using LAV Blot assay.

Figure 4.15 shows the sera incubated test strips of homologous and heterologous poxvirus prime /poxvirus boost regimens. In the homologous poxvirus prime/poxvirus boost regimens, rLSDV-Grttn prime/ rLSDV-Grttn boost regimen elicited antibody response for Gag (p55). rMVA-Grttn prime/ rMVA-Grttn boost regimen elicited antibody response for two Gag proteins (p55 and p25). In heterologous poxvirus prime/boost regimens, both rLSDV-Grttn prime/ rMVA-Grttn boost and rMVA-Grttn prime/ rLSDV-Grttn boost elicited antibody response to two Gag protein (p55 and p25), with relatively higher antibody responses (intensity of the band on test strips) compared to responses induced by homologous poxviruses. The non-specific antibodies binding to the test strip (position slightly above p68) were induced by vaccination regimens LSDV (wild-type and recombinant).

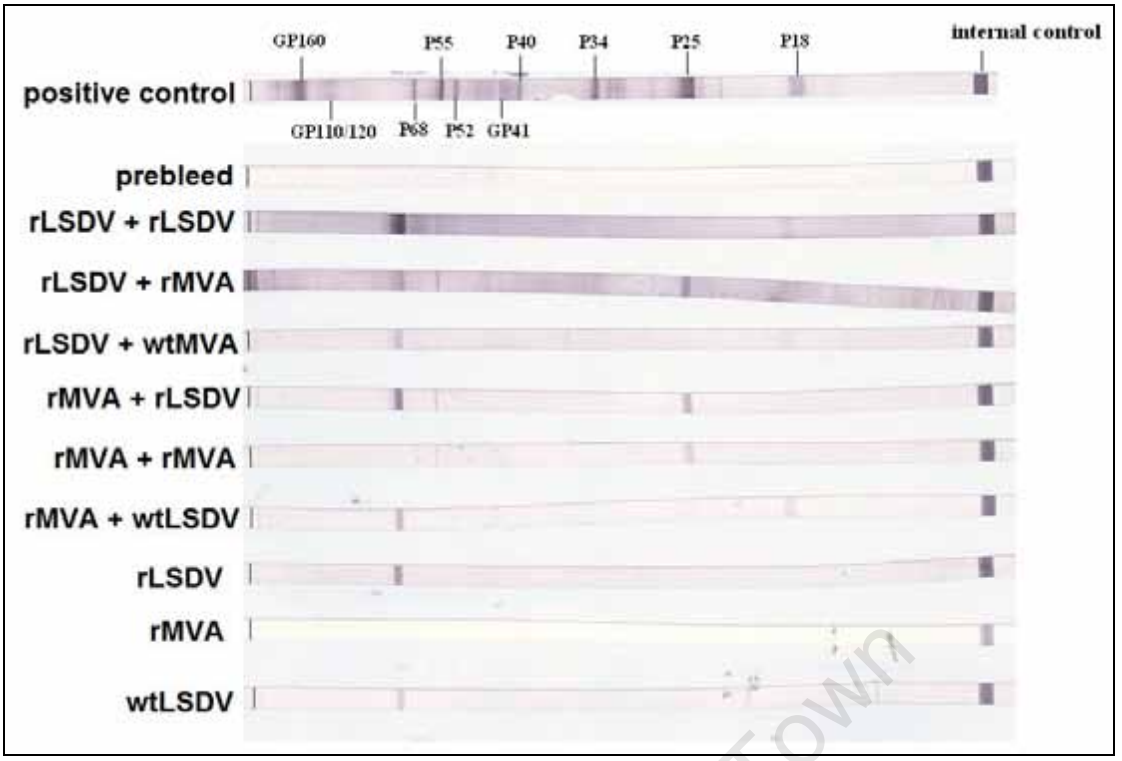

Figure 4.15 HIV-1 specific antibody in sera of mice vaccinated with single or poxviruses prime/boost vaccination regimens indicated in Figure 4.6. On day 40 sera were pooled from five mice per group. Sera obtained from the prebleed prior vaccination was included as negative control. The presence of HIV-1 antibody in these sera was detected using New LAV Blot Assay.

4.11 Discussion

The present study evaluated the safety of the Neethling strain of LSDV and the immunogenicity of rLSDV-Grttn. It has shown wtLSDV to be safe in two strains of immunocompromised mice with different immunodeficiency phenotypes. Similar evaluations of safety have been demonstrated for other poxvirus vectors, such as MVA in SCID mice (Hanke *et al.*, 2005; Wyatt *et al.*, 2004). MVA, inoculated into SCID mice up to a dose of 1 x 10^9 pfu, did not induce weight loss or other adverse effects (Wyatt *et al.*, 2004). Due to the limited ability of LSDV to be grown to high titres, it was not possible to use the equivalent dose of LSDV. Nevertheless, doses of 1 x 10^4 and 1 x 10^6 ffu of LSDV was not adverse to the well being of the mice and did not induce significant changes in weight compared to naive mice and PBS inoculated mice. This demonstrated that the vector is not pathogenic in these immunocompromised mice and suggests that the vector would be safe in immunocompromised humans, although further non-human primate and human clinical safety trials would be required to confirm this.

The immunogenicity of rLSDV-Grttn was evaluated after a single vaccination in BALB/c mice

and compared to the immunogenicity of rMVA-Grttn. In addition rLSDV-Grttn was evaluated in a DNA prime/poxvirus boost regimen and compared to the DNA prime/MVA boost regimen, which has been regimens extensively evaluated in different pre-clinical studies and ongoing clinical trials (Brave *et al.*, 2007; Burgers *et al.*, 2009; Goonetilleke *et al.*, 2006; Gudmundsdotter *et al.*, 2009; Guimaraes-Walker *et al.*, 2008; Hanke *et al.*, 2007; Sandstrom *et al.*, 2008). rLSDV-Grttn was also evaluated in heterologous prime/boost vaccination regimens with rMVA-Grttn to determine whether the dual poxvirus regimens were more efficacious than the DNA prime/ poxvirus boost regimens.

In this study we found that a single vaccination of 10^6 ffu of rLSDV-Grttn alone in mice was able to induce weak HIV-1 specific cellular responses in mice. A weak RT-specific CD8+ IFN-γ response and a weak CD4+ IL-2 response was elicited in rLSDV-Grttn vaccinated mice. In comparison, the same dose of rMVA-Grttn elicited broader and higher cellular immune responses compared to rLSDV-Grttn. Both RT- and Gag- specific IFN-γ responses were elicited in CD8+ and CD4+ T cells as well as IL-2 responses in CD4+ T cells. The DNA prime/poxvirus boost regimens resulted in improved immunogenicity compared to the single poxvirus vaccination regimens, as anticipated from the results of others (Hanke *et al.*, 2007; Im *et al.*, 2006; Shephard *et al.*, 2008). The cellular immune responses to the DNA prime were below the detection limit. A boost with rLSDV-Grttn induced a cumulative RT-specific IFN-γ response (607 net SFU/10^6 splenocytes) four times higher than that elicited by rLSDV-Grttn alone. However, the DNA/ rLSDV-Grttn regimen did not show any improved cellular immunogenicity over the DNA/ rMVA-Grttn regimen, which induced both Gag- and RT-specific IFN-γ responses with a cumulative IFN-γ response of 1529 net SFU/10^6 splenocytes and a cumulative IL-2 response of 245 net SFU/10^6 splenocytes.

Although the results of the present study suggest rMVA-Grttn induces superior immune responses to rLSDV-Grttn when used with a DNA prime, when rLSDV-Grttn and rMVA-Grttn were used in combination with one another in prime and boost vaccination regimens the magnitude of the cellular immune response was higher than that elicited by the DNA prime/rMVA-Grttn boost regimen. This was concluded from the finding that rLSDV-Grttn prime/rMVA-Grttn boost and the reverse regimen (rMVA-Grttn prime/rLSDV-Grttn boost) elicited cumulative Gag- and RT-specific IFN-γ responses that were, respectively, 2.0 and 1.7 times higher than those of the DNA prime/rMVA-Grttn regimen. For the induction of IL-2 secreting cells, rLSDV-Grttn prime/rMVA-Grttn boost and the reverse regimen (rMVA-Grttn prime/rLSDV-Grttn boost) elicited cumulative Gag- and RT-specific IL-2 responses that were,

respectively, 1.8 and 3.1 times higher than the cumulative Gag- and RT-specific IL-2 responses elicited by DNA prime/rMVA-Grttn.

It was concerning that the presence of rLSDV-Grttn in the vaccination regimens induced high non HIV-specific responses that could have contributed to the significantly better immunogenicity elicited by heterologous poxvirus prime/boost regimens over the DNA prime/rMVA-Grttn boost regimen. Since rLSDV-Grttn was propagated in bovine cells in the presence foetal bovine serum, we hypothesized that the non-specific responses could have resulted from mouse splenocytes responding to traces of bovine cellular molecules or foetal bovine serum present in the rLSDV-Grttn inoculum. The parallel experiments comparing ELISPOT assays done using foetal bovine serum containing medium (R10) and serum-free nutrient defined medium certainly suggest some contribution of responses to foetal bovine to high background responses as the high non-specific background responses observed in assays using R10 medium were lower in assays carried out in the presence of serum-free media. We have also shown in these parallel comparative experiments that, in the absence of high non-HIV specific responses, using serum-free medium, Gag- and RT- specific IFN-γ responses elicited by the rLSDV-Grttn prime/rMVA-Grttn boost and the reverse regimen did not diminish compared to the parallel counterpart experiment using foetal bovine serum in the medium. This suggests that the high immune responses elicited by the heterologous poxvirus prime/boost regimens were not false positive responses due to the presence of high non-specific background responses. Similar comparisons should be repeated for the DNA prime/ poxvirus boost regimens in order to further confirm their immunogenicity; however, due to funding constraints, we could only repeat the evaluation for the two most immunogenic regimens with the respective controls.

The comparisons between the different prime/ boost regimens evaluated in this study have revealed that the heterologous poxvirus prime/boost regimens are more immunogenic than the homologous poxvirus prime/boost regimens and the DNA prime/poxvirus boost regimens. The breadth and magnitude of the HIV-specific IFN-γ and IL-2 ELISPOT responses were comparable between the rLSDV-Grttn prime/rMVA-Grttn boost and the reverse regimen. Further investigation into the cellular immune responses elicited by the two heterologous prime/boost regimens revealed more detail and differences in their immunogenicity. The Gag- and RT-Pentamer binding assay detected a RT dominant CD8+ T cell response elicited by heterologous poxvirus prime-boost regimens, similar to the responses detected in IFN-γ ELISPOT assays. However, the percentages of Gag- or RT-specific CD8+ T cells detected using pentamer binding assays were 1.6-5 times higher than those of the HIV-specific CD8+ T cells detected using IFN-γ ELISPOT assays (see Table 3.1). Although the differences in the HIV-specific CD8+ T cells may be attributed to the different sensitivities of the assays, they may also

indicate that the vaccination regimens elicited Gag and RT-specific CD8+ T cells, other than IFN-γ producing CD8+ T cells. These may include other single or multiple cytokine producing HIV-specific CD8+ T cells. Unfortunately, further investigation was hampered by funding constraints. The evaluation of CD44 and CD107a/b expression on the surface of these Gag- and RT-specific CD8+ T cells gave an indirect indication of the activation and functional status of these cells. Over 98% of the Gag- or RT-specific CD8+ T cells elicited by the heterologous poxvirus prime/boost regimens were antigen-experienced and activated (CD44 surface expression). Over 70% of the Gag- or RT-specific CD8+ T cells elicited by the heterologous poxvirus prime/boost regimens had the capacity for degranulation (CD107a/b surface expression). An unexpected result was the lack of difference in surface expression of CD107a/b on the (Gag or RT) peptide stimulated Gag- or RT-specific CD8+ T cells and the non-stimulated Gag- or RT-specific CD8+ T cells. Further evaluation of the surface expression of CD107a/b on these splenocytes before stimulation is required to determine the initial level of CD107a/b. Again, this experiment was not repeated due to funding constraints. Although further evaluations would confirm the activation and functional status of these HIV-1 specific CD8+ T cells, the results from this study have given some insight into the potential activation and functionality of these HIV-1 specific CD8 T cells elicited by the rLSDV-Grttn prime/ rMVA-Grttn boost and the reverse regimen.

The use of different poxvirus vectors in prime/boost vaccination regimens have previously been demonstrated to be effective vaccination strategies (Cottingham *et al.*, 2006; Santra *et al.*, 2007). The order of the poxvirus vectors used in the prime/boost regimens affected the magnitude of the responses (Cottingham *et al.*, 2006). The observations made in the present study support the published observations to a degree. Although the magnitude and breadth of the Gag- and RT-specific cellular responses were comparable between the rLSDV-Grttn prime/ rMVA-Grttn boost and the reverse regimen, there was a difference in the quality of the response with respect to the level of IFN-γ production by the HIV-1 specific T cells in culture. The rMVA-Grttn prime/rLSDV-Grttn boost regimen elicited RT-specific CD4+ T cells with a 2.7 times higher capacity to produce IFN-γ compared to the RT-specific CD4+ T cells elicited by the reverse regimen. This suggests that the rMVA-Grttn prime/ rLSDV-Grttn boost regimen may be more immunogenic compared to the reverse regimen.

The association of non-neutralizing antibodies with the cellular immune response, through mechanisms such as complement binding to Fc receptors, has been highlighted in several studies (asa-Chapman *et al.*, 2005; Huber *et al.*, 2006; Huber *et al.*, 2008; Willey & asa-Chapman,

2008). Although HIV-1 envelope protein was not included in the rLSDV-Grttn vaccine, antibody specific to Grttn, or parts thereof, may be important in mediating HIV-1 specific cellular effector responses. We have demonstrated in this study that the rLSDV-Grttn prime/rMVA-Grttn boost regimen and the reverse regimen were able to induce Gag-specific antibody production.

In conclusion, the immunogenicity evaluation of rLSDV-Grttn in this study has revealed that rLSDV-Grttn has the potential to be used in heterologous poxvirus prime/boost regimens to induce a high magnitude and breadth of cellular immune responses, with the induction of surface molecules associated with immune cell activation and function. HIV-1 capsid protein-specific antibody was also produced. The rMVA-Grttn prime/rLSDV-Grttn boost regimen may be the better regimen due to the higher cytokine production per splenocyte induced by the regimen. The immunogenicity of the vaccination regimens should be further characterized to explore the potential of the rLSDV-Grttn prime/rMVA-Grttn boost and the reverse regimens.

Chapter 5: Final Discussion

The present study has evaluated LSDV as a potential vaccine vector for delivering HIV-1 antigens. We have demonstrated the Neethling strain of LSDV to be a safe, non-pathogenic live viral vector in mice with immunodeficiency phenotypes. LSDV did not induce any mortality, weight loss or other adverse effects in these immunocompromised mice. The non-pathogenicity of the virus in immunodeficient mice was not surprising when considering previous *in vitro* studies which showed that LSDV is replication-deficient in mammalian cells (Aspden *et al.*, 2003) and the fact that the host-restricted MVA is non-pathogenic in immunocompromised mice (Wyatt *et al.*, 2004). Our observations suggest that LSDV could be safely deployed in immunocompromised individuals in the event of large scale vaccination or as a therapeutic vaccine vector. Although further pre-clinical and clinical evaluations of LSDV in non-human primates and humans are needed to confirm the safety of the vector in populations comprising a high percentage of HIV-1 infected individuals, the encouraging pre-clinical safety data observed in this study does provide the motivation for further evaluation and development of LSDV as a vaccine vector.

The development of an effective HIV-1 vaccine is essential and a matter of urgency. It would be ideal to develop a universal HIV-1 vaccine that is effective in preventing infections of all the circulating HIV-1 strains in the world. However, the development of such a vaccine has proved to be very challenging due to the hypervariability of HIV-1(Burton *et al.*, 2004). It would be a more realistic goal to develop a HIV-1 vaccine that does not completely prevent infection but slows down the disease progression and minimises the chance of secondary infections. It is within our reach to develop such a vaccine that targets the most prevalent strain in a specific region to effectively control the epidemic in that region. rLSDV-Grttn constructed in this study was based on such a hypothesis. HIV-1 subtype C Gag, RT, Tat and Nef were used in the construction of Grttn (Burgers *et al.*, 2006; Williamson *et al.*, 2003), to produce a vaccine construct that specifically targets the HIV-1 subtype C strain that is currently the prevalent HIV-1 strain in Southern Africa. The vaccine antigens were selected to generate a broad HIV-1 specific cellular immune response. Gag-specific cellular immunity has been shown to correlate with slower disease progression in infected individuals (Pereyra *et al.*, 2008). Although immune responses to HIV-l regulatory proteins, such as Tat and Nef were not evaluated in this study, Tat and Nef were included in the vaccine antigen design to broaden the HIV-1 specific responses induced by the rLSDV-Grttn. Highly variable envelope proteins were excluded from the antigen selection in this study.

In this study a recombinant LSDV that expresses HIV-1 subtype C polyprotein Grttn (rLSDV-Grttn) was constructed. Stable expression of HIV-1 Grttn was detected from rLSDV-Grttn which was passaged 17 times.

As mentioned in the first chapter, DNA prime/poxvirus boost vaccination regimens, especially with MVA as a boost vaccination can induce high magnitudes of HIV-1 specific cellular immunity (Abaitua *et al.*, 2006; Amara *et al.*, 2002; Brave *et al.*, 2007; Burgers *et al.*, 2009; Gherardi *et al.*, 2004; Hanke *et al.*, 2007; Smith *et al.*, 2004). However, results from the STEP trial have highlighted the significance of pre-immunity to the Adenovirus vaccine vector and its possible negative effect on the effectiveness of the vaccine regimen (Corey *et al.*, 2009; McElrath *et al.*, 2008). Vaccination of individuals with pre-immunity to Adenovirus-5 vector increased their risk of infection with HIV-1 (Corey *et al.*, 2009; McElrath *et al.*, 2008). Whether pre-immunity to MVA/VV causes the equivalent increase in HIV-1 infection post recombinant MVA vaccination is not known. There is a significant portion of the population with pre-immunity to vaccinia virus or MVA, which could pose a potential problem to using MVA as a vector if pre-immunity is a problem. Nevertheless, there is a high probability that, with all of its advantages, MVA will be used in the future as a vector for a vaccine against an infectious disease or cancer. LSDV, which does not cross-react immunologically with MVA, could serve as an alternative vector. In this particular study, although rLSDV-Grttn was effective in boosting the HIV-1 specific immune response in the DNA prime/ rLSDV-Grttn boost vaccination regimen, rLSDV-Grttn failed to demonstrate superior immunogenicity to rMVA-Grttn in the comparative DNA prime/ rMVA-Grttn boost vaccination regimen. rLSDV-Grttn was, however, shown to be highly immunogenic when used in heterologous poxvirus prime/boost vaccination regimens with rMVA-Grttn. rLSDV-Grttn prime/ rMVA-Grttn boost and the reverse regimen induced 1.6-3.1 times higher magnitudes of HIV-1 Gag- and RT- specific immune responses compared to the DNA prime /rMVA-Grttn boost regimen. Both HIV-1 Gag- and RT-specific CD4+ and CD8+ T cell responses were induced by these vaccination regimens in mice. The HIV-1 specific cytotoxic CD8+ T cell response has been shown to correlate with the control of HIV-1 viraemia (Koup *et al.*, 1994; Miura *et al.*, 2009; Pereyra *et al.*, 2008). CD4+ T cell responses are essential in the production of effector and memory CD8+ T cell responses (Bevan, 2004; Sun *et al.*, 2004). Further evaluation in this study on the quality of the HIV-1 Gag and RT specific CD8+ T cell responses induced by these regimens indirectly demonstrated the activated, antigen-experienced status and cytolytic functional status by the presence of correlative surface molecules (CD44 and CD107a/b respectively) on these CD8+ T cells. HIV-1 Gag-specific antibodies were also induced by these vaccination regimens.

Preliminary evaluation of rLSDV-Grttn in a heterologous prime/boost vaccination regimen with SAAVI MVA-C in non-human primates has been carried out by Dr Wendy Burgers and Dr Gerald Chege in our department (unpublished results). Five rhesus macaques were vaccinated twice with recombinant MVA based vaccine, SAAVI MVA-C (Burgers *et al.*, 2008; Shephard *et al.*, 2008) expressing HIV-1 subtype C Grttn and envelope. After the priming of these animals with SAAVI-MVA-C two out of five macaques developed moderate IFN-γ ELISPOT responses directed to single HIV-1 genes. Sixty-six weeks after the second SAAVI-MVA prime macaques were boosted with a single dose of 1 x 10^6 ffu of rLSDV-Grttn. rLSDV-Grttn was able to boost HIV-1 specific IFN-γ ELISPOT responses in four out of five animals even though 3 of them had undetectable HIV-1 IFN-γ ELISPOT responses after two inoculations of SAAVI-MVA-C. Overall, both the magnitude and breadth of the IFN-γ ELISPOT responses were improved with rLSDV-Grttn boosting.

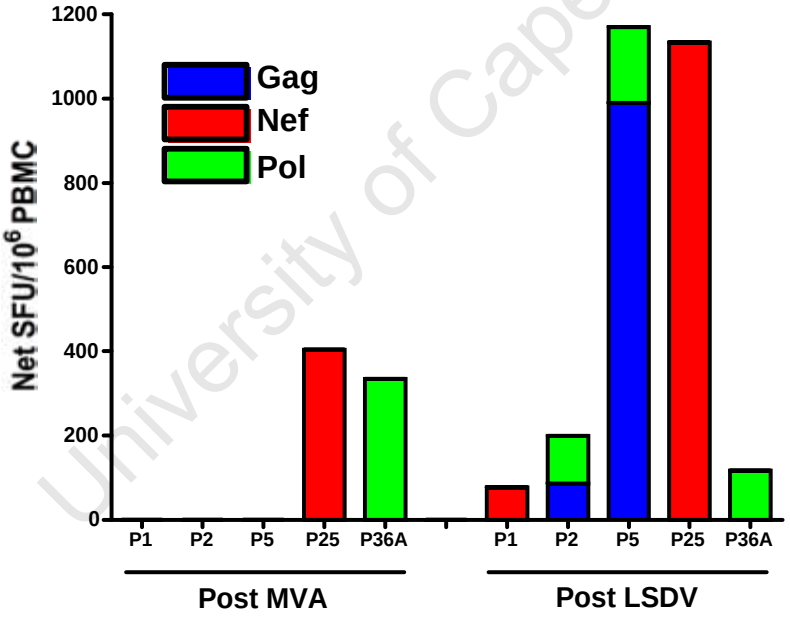

Figure 5.1 Cumulative HIV-1 specific IFN-γ ELISPOT responses of SAAVI-MVA-C prime/rLSDV-Grttn boost vaccination regimen in rhesus macaques. This figure shows responses after 2 inoculations of SAAVI-MVA-C priming (Post MVA) and those after a rLSDV-Grttn boost 66 weeks later (Post LSDV).

These observations, together with the mouse immunogenicity data demonstrate that heterologous prime/boost vaccination regimens using rLSDV-Grttn and rMVA-Grttn are promising potential vaccination regimens to use for the induction of potent HIV-1 specific cellular immune responses. Similar observations on the effectiveness of heterologous poxvirus

prime/boost vaccination regimens in inducing HIV-1 specific cellular immunity have been reported using fowlpoxvirus, MVA and vaccinia virus (Cottingham *et al.*, 2006; Ranasinghe *et al.*, 2006; Santra *et al.*, 2007).

The promising rMVA-Grttn prime/rLSDV-Grttn boost immunogenicity results in mice and non-human primates suggest that future LSDV based HIV-1 vaccine candidates could be integrated into existing prime-boost vaccination regimens. A vaccination regimen comprised of three different vaccine vectors expressing a common antigen has been shown to induce potent and long-lasting antigen-specific CD8+ T cells, especially CD8+ effector memory T cells (Masopust, 2009; Vezys *et al.*, 2009). The study deployed vesicular stomatitis virus (VSV) New Jersey strain, vaccinia virus and VSV Indiana strain in the regimen. The regimen induced antigen specific CD8+ T cells that comprised 90% of the circulating CD8+ T cells. More than 60% of the antigen-specific CD8+ T cells were retained 150 days post last vaccination (Masopust, 2009; Vezys *et al.*, 2009). However, a contrasting observation has been made by Vuola et al (2005) in an evaluation of a malaria vaccine. This study showed that the regimen comprised of DNA, MVA and fowlpox virus did not elicit an improved immune response in humans compared to the dual regimens of DNA prime/ MVA boost and fowlpox virus prime/MVA boost (Vuola *et al.*, 2005). Currently the prime and boost regimen with SAAVI-DNA-C and SAAVI-MVA-C ((Burgers *et al.*, 2008; Burgers *et al.*, 2009; Shephard *et al.*, 2008) is being evaluated in phase I clinical trials (HVTN 073). Both of the vaccine vectors in this trial were engineered to express HIV-1 subtype C Grttn and envelope protein. rLSDV-Grttn could be incorporated into this existing vaccination regimen as a third vaccine to potentially improve the immunogenicity of the present dual prime-boost regimen and induce a further broadening of the T cell repertoire. In the light of the recent promising preliminary results of the RV144 trial (Berkhout & Paxton, 2009), the importance of HIV-1 envelope protein in the protective efficacy against HIV-1 infection was emphasized. rLSDV-Grttn would need to be engineered further to incorporate the identical HIV-1 subtype C envelope gene to that of the HVTN 073 vaccines before it could be evaluated in a three vector prime-boost-boost vaccination regimen.

One of the concerns of using LSDV as a vaccine vector is that it has been passaged extensively in cells of bovine origin with bovine serum. Our immunogenicity data suggests that these products may be problematic and mice seem to develop responses to them which interfere in the assays used to measure specific responses. However most live attenuated viral vaccines are not purified extensively and would likely exhibit the same problems. For example the smallpox virus vaccine was made on cattle or sheep with limited purification. MVA based vaccines are made in eggs with limited purification. In addition these products may be regarded as a risk for

the prions associated with transmissible spongiform encephalopathies (TSEs) which are fatal neurodegenerative disorders (Yokoyama & Mohri, 2008). However the risk may be extremely low because there are stocks of LSDV vaccine which predate 1985 and BSE has not been reported in South Africa. Of importance there are a number of approaches that can be considered to reduce this risk. One of these involves cloning the poxviral DNA into a bacterium and then grow the vaccine in eggs or even better in a cell line which has a low risk of prion contamination. The second is to do extensive passages in such a cell line. The third is to harvest poxviral DNA and to rescue the DNA with another poxvirus after transfection into an acceptable cell line. The growth of LSDV in eggs was not investigated in this thesis. This would be worth investigating in the future.

However we can confidently conclude from the data presented that we have proved the hypothesis that LSDV has the potential to be a novel HIV-1 vaccine vector. It is safe in immunocompromised settings and rLSDV-Grttn has been demonstrated to be immunogenic in mice, especially in combination with rMVA-Grttn in prime-boost vaccination strategies. These observations provide motivation for the further optimization of LSDV virus production and the development of improved recombinant LSDV HIV-1 vaccines. Ultimately, it is conceivable that LSDV could be used in combination with other vectors as part of a dual or multi vaccine regimen to control the HIV-1 epidemic in Southern Africa.

References

1. **Abaitua, F., Rodriguez, J. R., Garzon, A., Rodriguez, D. & Esteban, M. (2006).**Improving recombinant MVA immune responses: potentiation of the immune responses to HIV-1 with MVA and DNA vectors expressing Env and the cytokines IL-12 and IFN-gamma. *Virus Res* **116**, 11-20.

2. **Abbas, A. K., Murphy, K. M. & Sher, A. (1996).**Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793.

3. **Alexander, R. A., Plowright, W. & Haig, D. A. (1957).**Cytopathic agents associated with LSD of cattle. *Bull Epizoot Dis Africa* **5**, 489-492.

4. **Ali, A. A., Esmat, M., Attia, H., Selim, A. & bdel-Hamid, Y. M. (1990).**Clinical and pathological studies on lumpy skin disease in Egypt. *Vet Rec* **127**, 549-550.

5. **Almeida, J. R., Price, D. A., Papagno, L., Arkoub, Z. A., Sauce, D., Bornstein, E., Asher, T. E., Samri, A., Schnuriger, A., Theodorou, I., Costagliola, D., Rouzioux, C., Agut, H., Marcelin, A. G., Douek, D., Autran, B. & Appay, V. (2007).**Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* **204**, 2473-2485.

6. **Amara, R. R., Nigam, P., Sharma, S., Liu, J. & Bostik, V. (2004).**Long-lived poxvirus immunity, robust CD4 help, and better persistence of CD4 than CD8 T cells. *J Virol* **78**, 3811-3816.

7. **Amara, R. R., Smith, J. M., Staprans, S. I., Montefiori, D. C., Villinger, F., Altman, J. D., O'Neil, S. P., Kozyr, N. L., Xu, Y., Wyatt, L. S., Earl, P. L., Herndon, J. G., McNicholl, J. M., McClure, H. M., Moss, B. & Robinson, H. L. (2002).**Critical role for Env as well as Gag-Pol in control of a simian-human immunodeficiency virus 89.6P challenge by a DNA prime/recombinant modified vaccinia virus Ankara vaccine. *J Virol* **76**, 6138-6146.

8. **Ami, Y., Izumi, Y., Matsuo, K., Someya, K., Kanekiyo, M., Horibata, S., Yoshino, N., Sakai, K., Shinohara, K., Matsumoto, S., Yamada, T., Yamazaki, S., Yamamoto, N. & Honda, M. (2005).**Priming-boosting vaccination with recombinant Mycobacterium bovis bacillus Calmette-Guerin and a nonreplicating vaccinia virus recombinant leads to long-lasting and effective immunity. *J Virol* **79**, 12871-12879.

9. **An, G., Hidaka, K. & Siminovitch, L. (1982).**Expression of bacterial beta-galactosidase in animal cells. *Mol Cell Biol* **2**, 1628-1632.

10. **Andre, S., Seed, B., Eberle, J., Schraut, W., Bultmann, A. & Haas, J. (1998).**Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J Virol* **72**, 1497-1503.

11. **Arora, S. K. (2002).**Analysis of intracellular cytokines using flowcytometry. *Methods Cell Sci* **24**, 37-40.

12. **asa-Chapman, M. M., Holuigue, S., Aubin, K., Wong, M., Jones, N. A., Cornforth, D., Pellegrino, P., Newton, P., Williams, I., Borrow, P. & McKnight, A. (2005).**Detection of antibody-dependent complement-mediated inactivation of both autologous and heterologous virus in primary human immunodeficiency virus type 1 infection. *J Virol* **79**, 2823-2830.

13. **Asher, D. M. (1999a).**Bovine sera used in the manufacture of biologicals: current concerns and policies of the U.S. Food and Drug Administration regarding the transmissible spongiform encephalopathies. *Dev Biol Stand* **99**, 41-44.

14. **Asher, D. M. (1999b).**The transmissible spongiform encephalopathy agents: concerns and responses of United States regulatory agencies in maintaining the safety of biologics. *Dev Biol Stand* **100**, 103-118.

15. **Asmuth, D. M., Brown, E. L., Dinubile, M. J., Sun, X., Del, R. C., Harro, C., Keefer, M. C., Kublin, J. G., Dubey, S. A., Kierstead, L. S., Casimiro, D. R., Shiver, J. W., Robertson, M. N., Quirk, E. K. & Mehrotra, D. V. (2009).**Comparative Cell-Mediated Immunogenicity of DNA/DNA, DNA/Adenovirus Type 5 (Ad5), or Ad5/Ad5 HIV-1 Clade B gag Vaccine Prime-Boost Regimens. *J Infect Dis*.

16. **Aspden, K., Passmore, J. A., Tiedt, F. & Williamson, A. L. (2003).**Evaluation of lumpy skin disease virus, a capripoxvirus, as a replication-deficient vaccine vector. *J Gen Virol* **84**, 1985-1996.

17. **Aspden, K., van Dijk, A. A., Bingham, J., Cox, D., Passmore, J. A. & Williamson, A. L. (2002).**Immunogenicity of a recombinant lumpy skin disease virus (neethling vaccine strain) expressing the rabies virus glycoprotein in cattle. *Vaccine* **20**, 2693-2701.

18. **Atkins, K. M., Thomas, L., Youker, R. T., Harriff, M. J., Pissani, F., You, H. & Thomas, G. (2008).**HIV-1 Nef binds PACS-2 to assemble a multikinase cascade that triggers major histocompatibility complex class I (MHC-I) down-regulation: analysis using short interfering RNA and knock-out mice. *J Biol Chem* **283**, 11772-11784.

19. **Babiuk, S., Baca-Estrada, M. E., Foldvari, M., Storms, M., Rabussay, D., Widera, G. & Babiuk, L. A. (2002a).**Electroporation improves the efficacy of DNA vaccines in large animals. *Vaccine* **20**, 3399-3408.

20. **Babiuk, S., Baca-Estrada, M. E., Pontarollo, R. & Foldvari, M. (2002b).**Topical delivery of plasmid DNA using biphasic lipid vesicles (Biphasix). *J Pharm Pharmacol* **54**, 1609-1614.

21. **Babiuk, S., Parkyn, G., Copps, J., Larence, J. E., Sabara, M. I., Bowden, T. R., Boyle, D. B. & Kitching, R. P. (2007).**Evaluation of an ovine testis cell line (OA3.Ts) for propagation of capripoxvirus isolates and development of an immunostaining technique for viral plaque visualization. *J Vet Diagn Invest* **19**, 486-491.

22. **Baldick, C. J., Jr., Keck, J. G. & Moss, B. (1992).**Mutational analysis of the core, spacer, and initiator regions of vaccinia virus intermediate-class promoters. *J Virol* **66**, 4710-4719.

23. **Banerjee, K., Andjelic, S., Klasse, P. J., Kang, Y., Sanders, R. W., Michael, E., Durso, R. J., Ketas, T. J., Olson, W. C. & Moore, J. P. (2009).**Enzymatic removal of mannose moieties can increase the immune response to HIV-1 gp120 *in vivo*. *Virology*.

24. **Banki, Z., Kacani, L., Rusert, P., Pruenster, M., Wilflingseder, D., Falkensammer, B., Stellbrink, H. J., van, L. J., Trkola, A., Dierich, M. P. & Stoiber, H. (2005).**Complement dependent trapping of infectious HIV in human lymphoid tissues. *AIDS* **19**, 481-486.

25. **Bansal, A., Gough, E., Ritter, D., Wilson, C., Mulenga, J., Allen, S. & Goepfert, P. A. (2006).**Group M-based HIV-1 Gag peptides are frequently targeted by T cells in chronically infected US and Zambian patients. *AIDS* **20**, 353-360.

26. **Barker, E., Mackewicz, C. E. & Levy, J. A. (1995).**Effects of TH1 and TH2 cytokines on CD8+ cell response against human immunodeficiency virus: implications for long-term survival. *Proc Natl Acad Sci U S A* **92**, 11135-11139.

27. **Barnard, B. J. H., Munz, E., Dumbell, K. & Prozesky, L. (1994).**Lumpy Skin Disease. In *Infectious Diseases of Livestock*, second edn, pp. 605-612. Edited by J. A. W. Coetzer & R. C. Tustin: Oxford University Press.

28. **Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., Lewis, M. G., Emini, E. A., Shiver, J. W. & Letvin, N. L. (2000).**Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**, 486-492.

29. **Baroudy, B. M., Venkatesan, S. & Moss, B. (1982).**Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. *Cell* **28**, 315-324.

30. **Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., xler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. (1983).**Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871.

31. **Baxby, D. & Paoletti, E. (1992).**Potential use of non-replicating vectors as recombinant vaccines. *Vaccine*

10, 8-9.

32. **Bejon, P., Ogada, E., Mwangi, T., Milligan, P., Lang, T., Fegan, G., Gilbert, S. C., Peshu, N., Marsh, K. & Hill, A. V. (2007).**Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS ONE* **2**, e707.

33. **Belyakov, I. M., Moss, B., Strober, W. & Berzofsky, J. A. (1999).**Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. *Proc Natl Acad Sci U S A* **96**, 4512-4517.

34. **Berhe, G., Minet, C., Le, G. C., Barrett, T., Ngangnou, A., Grillet, C., Libeau, G., Fleming, M., Black, D. N. & Diallo, A. (2003).**Development of a dual recombinant vaccine to protect small ruminants against peste-des-petits-ruminants virus and capripoxvirus infections. *J Virol* **77**, 1571-1577.

35. **Berkhout, B. & Paxton, W. A. (2009).**HIV vaccine: it may take two to tango, but no party time yet. *Retrovirology* **6**, 88.

36. **Berns, K. I. & Silverman, C. (1970).**Natural occurrence of cross-linked vaccinia virus deoxyribonucleic acid. *J Virol* **5**, 299-304.

37. **Berson, J. F., Long, D., Doranz, B. J., Rucker, J., Jirik, F. R. & Doms, R. W. (1996).**A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains. *J Virol* **70**, 6288-6295.

38. **Betts, M. R., Brenchley, J. M., Price, D. A., De Rosa, S. C., Douek, D. C., Roederer, M. & Koup, R. A. (2003).**Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* **281**, 65-78.

39. **Betts, M. R. & Koup, R. A. (2004).**Detection of T-cell degranulation: CD107a and b. *Methods Cell Biol* **75**, 497-512.

40. **Betts, M. R., Nason, M. C., West, S. M., De Rosa, S. C., Migueles, S. A., Abraham, J., Lederman, M. M., Benito, J. M., Goepfert, P. A., Connors, M., Roederer, M. & Koup, R. A. (2006).**HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* **107**, 4781-4789.

41. **Bevan, M. J. (2004).**Helping the CD8(+) T-cell response. *Nat Rev Immunol* **4**, 595-602.

42. **Binley, J. M., Wrin, T., Korber, B., Zwick, M. B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C. J. & Burton, D. R. (2004).**Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. *J Virol* **78**, 13232-13252.

43. **Boehm, U., Klamp, T., Groot, M. & Howard, J. C. (1997).**Cellular responses to interferon-gamma. *Annu Rev Immunol* **15**, 749-795.

44. **Bontjer, I., Land, A., Eggink, D., Verkade, E., Tuin, K., Baldwin, C., Pollakis, G., Paxton, W. A., Braakman, I., Berkhout, B. & Sanders, R. W. (2009).**Optimization of human immunodeficiency virus type 1 envelope glycoproteins with V1/V2 deleted, using virus evolution. *J Virol* **83**, 368-383.

45. **Boyer, J. D., Chattergoon, M. A., Ugen, K. E., Shah, A., Bennett, M., Cohen, A., Nyland, S., Lacy, K. E., Bagarazzi, M. L., Higgins, T. J., Baine, Y., Ciccarelli, R. B., Ginsberg, R. S., MacGregor, R. R. & Weiner, D. B. (1999).**Enhancement of cellular immune response in HIV-1 seropositive individuals: A DNA-based trial. *Clin Immunol* **90**, 100-107.

46. **Boyer, J. D., Cohen, A. D., Vogt, S., Schumann, K., Nath, B., Ahn, L., Lacy, K., Bagarazzi, M. L., Higgins, T. J., Baine, Y., Ciccarelli, R. B., Ginsberg, R. S., MacGregor, R. R. & Weiner, D. B. (2000).**Vaccination of seronegative volunteers with a human immunodeficiency virus type 1 env/rev DNA vaccine induces antigen-specific proliferation and lymphocyte production of beta-chemokines. *J Infect Dis* **181**, 476-483.

47. **Boyer, J. D., Robinson, T. M., Kutzler, M. A., Parkinson, R., Calarota, S. A., Sidhu, M. K., Muthumani, K., Lewis, M., Pavlakis, G., Felber, B. & Weiner, D. (2005).**SIV DNA vaccine co-

administered with IL-12 expression plasmid enhances CD8 SIV cellular immune responses in cynomolgus macaques. *J Med Primatol* **34**, 262-270.

48. **Boyle, D. B. (2007).**Genus *Avipoxvirus*. In *Poxviruses*, pp. 217-251. Edited by A. A. Mercer, A. Schmidt & O. Weber: Birkha"user.

49. **Boyle, D. B. & Coupar, B. E. (1988).**A dominant selectable marker for the construction of recombinant poxviruses. *Gene* **65**, 123-128.

50. **Brave, A., Boberg, A., Gudmundsdotter, L., Rollman, E., Hallermalm, K., Ljungberg, K., Blomberg, P., Stout, R., Paulie, S., Sandstrom, E., Biberfeld, G., Earl, P., Moss, B., Cox, J. H. & Wahren, B. (2007).**A new multi-clade DNA prime/recombinant MVA boost vaccine induces broad and high levels of HIV-1-specific CD8(+) T-cell and humoral responses in mice. *Mol Ther* **15**, 1724-1733.

51. **Bridgeman, A., Roshorm, Y., Lockett, L. J., Xu, Z. Z., Hopkins, R., Shaw, J., Both, G. W. & Hanke, T. (2009).**Ovine atadenovirus, a novel and highly immunogenic vector in prime-boost studies of a candidate HIV-1 vaccine. *Vaccine*.

52. **Brooimans, R. A., Boyce, C. S., Popma, J., Broyles, D. A., Gratama, J. W., Southwick, P. C. & Keeney, M. (2008).**Analytical performance of a standardized single-platform MHC tetramer assay for the identification and enumeration of CMV-specific CD8+ T lymphocytes. *Cytometry A* **73**, 992-1000.

53. **Brown, E., Senkevich, T. G. & Moss, B. (2006a).**Vaccinia virus F9 virion membrane protein is required for entry but not virus assembly, in contrast to the related L1 protein. *J Virol* **80**, 9455-9464.

54. **Brown, S. A., Slobod, K. S., Surman, S., Zirkel, A., Zhan, X. & Hurwitz, J. L. (2006b).**Individual HIV type 1 envelope-specific T cell responses and epitopes do not segregate by virus subtype. *AIDS Res Hum Retroviruses* **22**, 188-194.

55. **Broyles, S. S. (2003).**Vaccinia virus transcription. *J Gen Virol* **84**, 2293-2303.

56. **Buchbinder, S. P., Katz, M. H., Hessol, N. A., O'Malley, P. M. & Holmberg, S. D. (1994).**Long-term HIV-1 infection without immunologic progression. *AIDS* **8**, 1123-1128.

57. **Buchbinder, S. P., Mehrotra, D. V., Duerr, A., Fitzgerald, D. W., Mogg, R., Li, D., Gilbert, P. B., Lama, J. R., Marmor, M., Del, R. C., McElrath, M. J., Casimiro, D. R., Gottesdiener, K. M., Chodakewitz, J. A., Corey, L. & Robertson, M. N. (2008).**Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* **372**, 1881-1893.

58. **Budd, R. C., Cerottini, J. C., Horvath, C., Bron, C., Pedrazzini, T., Howe, R. C. & MacDonald, H. R. (1987).**Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J Immunol* **138**, 3120-3129.

59. **Buller, R. M., Smith, G. L., Cremer, K., Notkins, A. L. & Moss, B. (1985).**Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* **317**, 813-815.

60. **Burgers, W. A., Chege, G. K., Muller, T. L., Van Harmelen, J. H., Khoury, G., Shephard, E. G., Gray, C. M., Williamson, C. & Williamson, A. L. (2009).**Broad, high-magnitude and multifunctional CD4+ and CD8+ T-cell responses elicited by a DNA and modified vaccinia Ankara vaccine containing human immunodeficiency virus type 1 subtype C genes in baboons. *J Gen Virol* **90**, 468-480.

61. **Burgers, W. A., Shephard, E., Monroe, J. E., Greenhalgh, T., Binder, A., Hurter, E., Van Harmelen, J. H., Williamson, C. & Williamson, A. L. (2008).**Construction, characterization, and immunogenicity of a multigene modified vaccinia Ankara (MVA) vaccine based on HIV type 1 subtype C. *AIDS Res Hum Retroviruses* **24**, 195-206.

62. **Burgers, W. A., Van Harmelen, J. H., Shephard, E., Adams, C., Mgwebi, T., Bourn, W., Hanke, T., Williamson, A. L. & Williamson, C. (2006).**Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. *J Gen Virol* **87**, 399-410.

63. **Burton, D. R., Barbas, C. F., III, Persson, M. A., Koenig, S., Chanock, R. M. & Lerner, R. A.**

(1991).A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci U S A* **88**, 10134-10137.

64. **Burton, D. R., Desrosiers, R. C., Doms, R. W., Koff, W. C., Kwong, P. D., Moore, J. P., Nabel, G. J., Sodroski, J., Wilson, I. A. & Wyatt, R. T. (2004).**HIV vaccine design and the neutralizing antibody problem. *Nat Immunol* **5**, 233-236.

65. **Cairns, J. (1960).**The initiation of vaccinia infection. *Virology* **11**, 603-623.

66. **Calarese, D. A., Lee, H. K., Huang, C. Y., Best, M. D., Astronomo, R. D., Stanfield, R. L., Katinger, H., Burton, D. R., Wong, C. H. & Wilson, I. A. (2005).**Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. *Proc Natl Acad Sci U S A* **102**, 13372-13377.

67. **Cardoso, R. M., Zwick, M. B., Stanfield, R. L., Kunert, R., Binley, J. M., Katinger, H., Burton, D. R. & Wilson, I. A. (2005).**Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity* **22**, 163-173.

68. **Carn, V. M. (1993).**Control of capripoxvirus infections. *Vaccine* **11**, 1275-1279.

69. **Carn, V. M. & Kitching, R. P. (1995).**An investigation of possible routes of transmission of lumpy skin disease virus (Neethling). *Epidemiol Infect* **114**, 219-226.

70. **Carroll, M. W. & Moss, B. (1997).**Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology* **238**, 198-211.

71. **Carter, G. C., Rodger, G., Murphy, B. J., Law, M., Krauss, O., Hollinshead, M. & Smith, G. L. (2003).**Vaccinia virus cores are transported on microtubules. *J Gen Virol* **84**, 2443-2458.

72. **Casimiro, D. R., Chen, L., Fu, T. M., Evans, R. K., Caulfield, M. J., Davies, M. E., Tang, A., Chen, M., Huang, L., Harris, V., Freed, D. C., Wilson, K. A., Dubey, S., Zhu, D. M., Nawrocki, D., Mach, H., Troutman, R., Isopi, L., Williams, D., Hurni, W., Xu, Z., Smith, J. G., Wang, S., Liu, X., Guan, L., Long, R., Trigona, W., Heidecker, G. J., Perry, H. C., Persaud, N., Toner, T. J., Su, Q., Liang, X., Youil, R., Chastain, M., Bett, A. J., Volkin, D. B., Emini, E. A. & Shiver, J. W. (2003).**Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. *J Virol* **77**, 6305-6313.

73. **Casimiro, D. R., Wang, F., Schleif, W. A., Liang, X., Zhang, Z. Q., Tobery, T. W., Davies, M. E., McDermott, A. B., O'Connor, D. H., Fridman, A., Bagchi, A., Tussey, L. G., Bett, A. J., Finnefrock, A. C., Fu, T. M., Tang, A., Wilson, K. A., Chen, M., Perry, H. C., Heidecker, G. J., Freed, D. C., Carella, A., Punt, K. S., Sykes, K. J., Huang, L., Ausensi, V. I., Bachinsky, M., Sadasivan-Nair, U., Watkins, D. I., Emini, E. A. & Shiver, J. W. (2005).**Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with dna and recombinant adenoviral vaccine vectors expressing Gag. *J Virol* **79**, 15547-15555.

74. **Chan, D. C. & Kim, P. S. (1998).**HIV entry and its inhibition. *Cell* **93**, 681-684.

75. **Chattopadhyay, P. K., Melenhorst, J. J., Ladell, K., Gostick, E., Scheinberg, P., Barrett, A. J., Wooldridge, L., Roederer, M., Sewell, A. K. & Price, D. A. (2008).**Techniques to improve the direct ex vivo detection of low frequency antigen-specific CD8+ T cells with peptide-major histocompatibility complex class I tetramers. *Cytometry A* **73**, 1001-1009.

76. **Chaudhri, G., Panchanathan, V., Bluethmann, H. & Karupiah, G. (2006).**Obligatory requirement for antibody in recovery from a primary poxvirus infection. *J Virol* **80**, 6339-6344.

77. **Chege, G. K., Shephard, E. G., Meyers, A., van, H. J., Williamson, C., Lynch, A., Gray, C. M., Rybicki, E. P. & Williamson, A. L. (2008).**HIV-1 subtype C Pr55gag virus-like particle vaccine efficiently boosts baboons primed with a matched DNA vaccine. *J Gen Virol* **89**, 2214-2227.

78. **Chen, X., Rock, M. T., Hammonds, J., Tartaglia, J., Shintani, A., Currier, J., Slike, B., Crowe, J. E., Jr., Marovich, M. & Spearman, P. (2005).**Pseudovirion particle production by live poxvirus human

immunodeficiency virus vaccine vector enhances humoral and cellular immune responses. *J Virol* **79**, 5537-5547.

79. **Cheng, L., Fu, J., Tsukamoto, A. & Hawley, R. G. (1996).**Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. *Nat Biotechnol* **14**, 606-609.

80. **Chihota, C. M., Rennie, L. F., Kitching, R. P. & Mellor, P. S. (2001).**Mechanical transmission of lumpy skin disease virus by Aedes aegypti (Diptera: Culicidae). *Epidemiol Infect* **126**, 317-321.

81. **Child, S. J., Palumbo, G. J., Buller, R. M. & Hruby, D. E. (1990).**Insertional inactivation of the large subunit of ribonucleotide reductase encoded by vaccinia virus is associated with reduced virulence *in vivo*. *Virology* **174**, 625-629.

82. **Chiu, W. L., Lin, C. L., Yang, M. H., Tzou, D. L. & Chang, W. (2007).**Vaccinia virus 4c (A26L) protein on intracellular mature virus binds to the extracellular cellular matrix laminin. *J Virol* **81**, 2149-2157.

83. **Chun, T. W., Engel, D., Berrey, M. M., Shea, T., Corey, L. & Fauci, A. S. (1998).**Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A* **95**, 8869-8873.

84. **Clerici, M., Hakim, F. T., Venzon, D. J., Blatt, S., Hendrix, C. W., Wynn, T. A. & Shearer, G. M. (1993).**Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. *J Clin Invest* **91**, 759-765.

85. **Cooney, E. L., Collier, A. C., Greenberg, P. D., Coombs, R. W., Zarling, J., Arditti, D. E., Hoffman, M. C., Hu, S. L. & Corey, L. (1991).**Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. *Lancet* **337**, 567-572.

86. **Cooney, E. L., McElrath, M. J., Corey, L., Hu, S. L., Collier, A. C., Arditti, D., Hoffman, M., Coombs, R. W., Smith, G. E. & Greenberg, P. D. (1993).**Enhanced immunity to human immunodeficiency virus (HIV) envelope elicited by a combined vaccine regimen consisting of priming with a vaccinia recombinant expressing HIV envelope and boosting with gp160 protein. *Proc Natl Acad Sci U S A* **90**, 1882-1886.

87. **Corey, L., McElrath, M. J. & Kublin, J. G. (2009).**Post-step modifications for research on HIV vaccines. *AIDS* **23**, 3-8.

88. **Cottingham, M. G., Andersen, R. F., Spencer, A. J., Saurya, S., Furze, J., Hill, A. V. & Gilbert, S. C. (2008).**Recombination-mediated genetic engineering of a bacterial artificial chromosome clone of modified vaccinia virus Ankara (MVA). *PLoS ONE* **3**, e1638.

89. **Cottingham, M. G., van, M. A., Zago, M., Newton, A. T., Anderson, R. J., Howard, M. K., Schneider, J. & Skinner, M. A. (2006).**Different levels of immunogenicity of two strains of Fowlpox virus as recombinant vaccine vectors eliciting T-cell responses in heterologous prime-boost vaccination strategies. *Clin Vaccine Immunol* **13**, 747-757.

90. **Cox, K. S., Clair, J. H., Prokop, M. T., Sykes, K. J., Dubey, S. A., Shiver, J. W., Robertson, M. N. & Casimiro, D. R. (2008).**DNA gag/adenovirus type 5 (Ad5) gag and Ad5 gag/Ad5 gag vaccines induce distinct T-cell response profiles. *J Virol* **82**, 8161-8171.

91. **D'Souza, W. N., Schluns, K. S., Masopust, D. & Lefrancois, L. (2002).**Essential role for IL-2 in the regulation of antiviral extralymphoid CD8 T cell responses. *J Immunol* **168**, 5566-5572.

92. **Dagert, M. & Ehrlich, S. D. (1979).**Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. *Gene* **6**, 23-28.

93. **Dale, C. J., De, R. R., Stratov, I., Chea, S., Montefiori, D. C., Thomson, S., Ramshaw, I. A., Coupar, B. E., Boyle, D. B., Law, M. & Kent, S. J. (2004).**Efficacy of DNA and fowlpox virus priming/boosting vaccines for simian/human immunodeficiency virus. *J Virol* **78**, 13819-13828.

94. **Dales, S. & Siminovitch, L. (1961).**The development of vaccinia virus in Earle's L strain cells as examined by electron microscopy. *J Biophys Biochem Cytol* **10**, 475-503.

95. **Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K. & Desrosiers, R. C. (1992).**Protective effects

of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258**, 1938-1941.

96. **Davies, F. G. (1981).**Attempted isolation of arboviruses from wild ruminants in Kenya. *Trop Anim Health Prod* **13**, 116.

97. **Davies, F. G. (1991).**Lumpy skin disease, an African capripox virus disease of cattle. *Br Vet J* **147**, 489-503.

98. **Davison, A. J. & Moss, B. (1989).**Structure of vaccinia virus early promoters. *J Mol Biol* **210**, 749-769.

99. **de Bruyn G., Rossini, A. J., Chiu, Y. L., Holman, D., Elizaga, M. L., Frey, S. E., Burke, D., Evans, T. G., Corey, L. & Keefer, M. C. (2004).**Safety profile of recombinant canarypox HIV vaccines. *Vaccine* **22**, 704-713.

100. **de Quadros, C. A., Hersh, B. S., Nogueira, A. C., Carrasco, P. A. & da Silveira, C. M. (1998).**Measles eradication: experience in the Americas. *Bull World Health Organ* **76 Suppl 2**, 47-52.

101. **Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di, M. P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R. & Landau, N. R. (1996).**Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661-666.

102. **DeVico, A. L., Fouts, T. R., Shata, M. T., Kamin-Lewis, R., Lewis, G. K. & Hone, D. M. (2002).**Development of an oral prime-boost strategy to elicit broadly neutralizing antibodies against HIV-1. *Vaccine* **20**, 1968-1974.

103. **Diallo, A. & Viljoen, G. J. (2007).**Genus *Capripoxvirus*. In *Poxviruses*, pp. 167-181. Edited by A. A. Mercer, A. Schmidt & O. Weber: Birkha"user.

104. **Doan, L. X., Li, M., Chen, C. & Yao, Q. (2005).**Virus-like particles as HIV-1 vaccines. *Rev Med Virol* **15**, 75-88.

105. **Domi, A. & Moss, B. (2002).**Cloning the vaccinia virus genome as a bacterial artificial chromosome in Escherichia coli and recovery of infectious virus in mammalian cells. *Proc Natl Acad Sci U S A* **99**, 12415-12420.

106. **Douek, D. C., Kwong, P. D. & Nabel, G. J. (2006).**The rational design of an AIDS vaccine. *Cell* **124**, 677-681.

107. **Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. & Paxton, W. A. (1996).**HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667-673.

108. **Dutton, R. W., Bradley, L. M. & Swain, S. L. (1998).**T cell memory. *Annu Rev Immunol* **16**, 201-223.

109. **Eckert, D., Williams, O., Meseda, C. A. & Merchlinsky, M. (2005).**Vaccinia virus nicking-joining enzyme is encoded by K4L (VACWR035). *J Virol* **79**, 15084-15090.

110. **Elango, N., Prince, G. A., Murphy, B. R., Venkatesan, S., Chanock, R. M. & Moss, B. (1986).**Resistance to human respiratory syncytial virus (RSV) infection induced by immunization of cotton rats with a recombinant vaccinia virus expressing the RSV G glycoprotein. *Proc Natl Acad Sci U S A* **83**, 1906-1910.

111. **Evans, D. T., Bricker, J. E., Sanford, H. B., Lang, S., Carville, A., Richardson, B. A., Piatak, M., Jr., Lifson, J. D., Mansfield, K. G. & Desrosiers, R. C. (2005).**Immunization of macaques with single-cycle simian immunodeficiency virus (SIV) stimulates diverse virus-specific immune responses and reduces viral loads after challenge with SIVmac239. *J Virol* **79**, 7707-7720.

112. **Falkner, F. G. & Moss, B. (1988).**Escherichia coli gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J Virol* **62**, 1849-1854.

113. **Feng, Y., Broder, C. C., Kennedy, P. E. & Berger, E. A. (1996).**HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872-877.

114. **Ferrantelli, F., Kitabwalla, M., Rasmussen, R. A., Cao, C., Chou, T. C., Katinger, H., Stiegler, G., Cavacini, L. A., Bai, Y., Cotropia, J., Ugen, K. E. & Ruprecht, R. M. (2004a).**Potent cross-group neutralization of primary human immunodeficiency virus isolates with monoclonal antibodies--implications for acquired immunodeficiency syndrome vaccine. *J Infect Dis* **189**, 71-74.

115. **Ferrantelli, F., Rasmussen, R. A., Buckley, K. A., Li, P. L., Wang, T., Montefiori, D. C., Katinger, H., Stiegler, G., Anderson, D. C., McClure, H. M. & Ruprecht, R. M. (2004b).**Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies. *J Infect Dis* **189**, 2167-2173.

116. **Flynn, N. M., Forthal, D. N., Harro, C. D., Judson, F. N., Mayer, K. H. & Para, M. F. (2005).**Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis* **191**, 654-665.

117. **Foo, C. H., Lou, H., Whitbeck, J. C., Ponce-de-Leon, M., Atanasiu, D., Eisenberg, R. J. & Cohen, G. H. (2009).**Vaccinia virus L1 binds to cell surfaces and blocks virus entry independently of glycosaminoglycans. *Virology* **385**, 368-382.

118. **Frischknecht, F., Cudmore, S., Moreau, V., Reckmann, I., Rottger, S. & Way, M. (1999a).**Tyrosine phosphorylation is required for actin-based motility of vaccinia but not Listeria or Shigella. *Curr Biol* **9**, 89-92.

119. **Frischknecht, F., Moreau, V., Rottger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G. & Way, M. (1999b).**Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. *Nature* **401**, 926-929.

120. **Fukazawa, Y., Miyake, A., Ibuki, K., Inaba, K., Saito, N., Motohara, M., Horiuchi, R., Himeno, A., Matsuda, K., Matsuyama, M., Takahashi, H., Hayami, M., Igarashi, T. & Miura, T. (2008).**Small intestine CD4+ T cells are profoundly depleted during acute simian-human immunodeficiency virus infection, regardless of viral pathogenicity. *J Virol* **82**, 6039-6044.

121. **Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Zolla-Pazner, S., Leibowitch, J. & Popovic, M. (1983).**Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**, 865-867.

122. **Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M., Shaw, G. M., Sharp, P. M. & Hahn, B. H. (1999).**Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**, 436-441.

123. **Garcia, P. M., Kalish, L. A., Pitt, J., Minkoff, H., Quinn, T. C., Burchett, S. K., Kornegay, J., Jackson, B., Moye, J., Hanson, C., Zorrilla, C. & Lew, J. F. (1999).**Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. Women and Infants Transmission Study Group. *N Engl J Med* **341**, 394-402.

124. **Gauduin, M. C., Parren, P. W., Weir, R., Barbas, C. F., Burton, D. R. & Koup, R. A. (1997).**Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1. *Nat Med* **3**, 1389-1393.

125. **Genesca, M., Skinner, P. J., Bost, K. M., Lu, D., Wang, Y., Rourke, T. L., Haase, A. T., McChesney, M. B. & Miller, C. J. (2008).**Protective attenuated lentivirus immunization induces SIV-specific T cells in the genital tract of rhesus monkeys. *Mucosal Immunol* **1**, 219-228.

126. **Ghanekar, S. A. & Maecker, H. T. (2003).**Cytokine flow cytometry: multiparametric approach to immune function analysis. *Cytotherapy* **5**, 1-6.

127. **Gherardi, M. M., Perez-Jimenez, E., Najera, J. L. & Esteban, M. (2004).**Induction of HIV immunity in the genital tract after intranasal delivery of a MVA vector: enhanced immunogenicity after DNA prime-modified vaccinia virus Ankara boost immunization schedule. *J Immunol* **172**, 6209-6220.

128. **Girard, M. P., Osmanov, S. K. & Kieny, M. P. (2006).**A review of vaccine research and development: the human immunodeficiency virus (HIV). *Vaccine* **24**, 4062-4081.

129. **Giri, M., Ugen, K. E. & Weiner, D. B. (2004).**DNA vaccines against human immunodeficiency virus type

1 in the past decade. *Clin Microbiol Rev* **17**, 370-389.

130. **Giuliano, M. & Vella, S. (2007).**Inequalities in health: access to treatment for HIV/AIDS. *Ann Ist Super Sanita* **43**, 313-316.

131. **Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P. & Paoletti, E. (1990).**The complete DNA sequence of vaccinia virus. *Virology* **179**, 247-263.

132. **Goepfert, P. A., Lumm, W., Farmer, P., Matthews, P., Prendergast, A., Carlson, J. M., Derdeyn, C. A., Tang, J., Kaslow, R. A., Bansal, A., Yusim, K., Heckerman, D., Mulenga, J., Allen, S., Goulder, P. J. & Hunter, E. (2008).**Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. *J Exp Med* **205**, 1009-1017.

133. **Gomez, C. E., Najera, J. L., Krupa, M. & Esteban, M. (2008).**The poxvirus vectors MVA and NYVAC as gene delivery systems for vaccination against infectious diseases and cancer. *Curr Gene Ther* **8**, 97-120.

134. **Goonetilleke, N., Moore, S., Dally, L., Winstone, N., Cebere, I., Mahmoud, A., Pinheiro, S., Gillespie, G., Brown, D., Loach, V., Roberts, J., Guimaraes-Walker, A., Hayes, P., Loughran, K., Smith, C., De, B. J., Verlinde, C., Vooijs, D., Schmidt, C., Boaz, M., Gilmour, J., Fast, P., Dorrell, L., Hanke, T. & McMichael, A. J. (2006).**Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes. *J Virol* **80**, 4717-4728.

135. **Gotte, M., Li, X. & Wainberg, M. A. (1999).**HIV-1 reverse transcription: a brief overview focused on structure-function relationships among molecules involved in initiation of the reaction. *Arch Biochem Biophys* **365**, 199-210.

136. **Graham, B. S., Belshe, R. B., Clements, M. L., Dolin, R., Corey, L., Wright, P. F., Gorse, G. J., Midthun, K., Keefer, M. C., Roberts, N. J., Jr. & . (1992).**Vaccination of vaccinia-naive adults with human immunodeficiency virus type 1 gp160 recombinant vaccinia virus in a blinded, controlled, randomized clinical trial. The AIDS Vaccine Clinical Trials Network. *J Infect Dis* **166**, 244-252.

137. **Gray, R. H., Wawer, M. J., Brookmeyer, R., Sewankambo, N. K., Serwadda, D., Wabwire-Mangen, F., Lutalo, T., Li, X., Vancott, T. & Quinn, T. C. (2001).**Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet* **357**, 1149-1153.

138. **Greenough, T. C., Cunningham, C. K., Muresan, P., McManus, M., Persaud, D., Fenton, T., Barker, P., Gaur, A., Panicali, D., Sullivan, J. L. & Luzuriaga, K. (2008).**Safety and immunogenicity of recombinant poxvirus HIV-1 vaccines in young adults on highly active antiretroviral therapy. *Vaccine* **26**, 6883-6893.

139. **Greth, A., Gourreau, J. M., Vassart, M., Nguyen, B., V, Wyers, M. & Lefevre, P. C. (1992).**Capripoxvirus disease in an Arabian oryx (Oryx leucoryx) from Saudi Arabia. *J Wildl Dis* **28**, 295-300.

140. **Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., McNeil, A. & Dandekar, S. (2003).**Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* **77**, 11708-11717.

141. **Gubser, C., Hue, S., Kellam, P. & Smith, G. L. (2004).**Poxvirus genomes: a phylogenetic analysis. *J Gen Virol* **85**, 105-117.

142. **Gudmundsdotter, L., Nilsson, C., Brave, A., Hejdeman, B., Earl, P., Moss, B., Robb, M., Cox, J., Michael, N., Marovich, M., Biberfeld, G., Sandstrom, E. & Wahren, B. (2009).**Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity. *Vaccine* **27**, 4468-4474.

143. **Guimaraes-Walker, A., Mackie, N., McCormack, S., Hanke, T., Schmidt, C., Gilmour, J., Barin, B., McMichael, A., Weber, J., Legg, K., Babiker, A., Hayes, P., Gotch, F., Smith, C., Dally, L., Dorrell, L., Cebere, I., Kay, R., Winstone, N., Moore, S., Goonetilleke, N. & Fast, P. (2008).**Lessons from IAVI-006, a phase I clinical trial to evaluate the safety and immunogenicity of the pTHr.HIVA DNA and

MVA.HIVA vaccines in a prime-boost strategy to induce HIV-1 specific T-cell responses in healthy volunteers. *Vaccine* **26**, 6671-6677.

144. **Gunasinghe, S. K., Hubbs, A. E. & Wright, C. F. (1998).**A vaccinia virus late transcription factor with biochemical and molecular identity to a human cellular protein. *J Biol Chem* **273**, 27524-27530.

145. **Gupta, K., Hudgens, M., Corey, L., McElrath, M. J., Weinhold, K., Montefiori, D. C., Gorse, G. J., Frey, S. E., Keefer, M. C., Evans, T. G., Dolin, R., Schwartz, D. H., Harro, C., Graham, B., Spearman, P. W., Mulligan, M. & Goepfert, P. (2002).**Safety and immunogenicity of a high-titered canarypox vaccine in combination with rgp120 in a diverse population of HIV-1-uninfected adults: AIDS Vaccine Evaluation Group Protocol 022A. *J Acquir Immune Defic Syndr* **29**, 254-261.

146. **Gupta, S. B., Jacobson, L. P., Margolick, J. B., Rinaldo, C. R., Phair, J. P., Jamieson, B. D., Mehrotra, D. V., Robertson, M. N. & Straus, W. L. (2007).**Estimating the benefit of an HIV-1 vaccine that reduces viral load set point. *J Infect Dis* **195**, 546-550.

147. **Hammarlund, E., Lewis, M. W., Hansen, S. G., Strelow, L. I., Nelson, J. A., Sexton, G. J., Hanifin, J. M. & Slifka, M. K. (2003).**Duration of antiviral immunity after smallpox vaccination. *Nat Med* **9**, 1131-1137.

148. **Hammonds, J., Chen, X., Fouts, T., DeVico, A., Montefiori, D. & Spearman, P. (2005).**Induction of neutralizing antibodies against human immunodeficiency virus type 1 primary isolates by Gag-Env pseudovirion immunization. *J Virol* **79**, 14804-14814.

149. **Hanke, T., Goonetilleke, N., McMichael, A. J. & Dorrell, L. (2007).**Clinical experience with plasmid DNA- and modified vaccinia virus Ankara-vectored human immunodeficiency virus type 1 clade A vaccine focusing on T-cell induction. *J Gen Virol* **88**, 1-12.

150. **Hanke, T., McMichael, A. J., Dennis, M. J., Sharpe, S. A., Powell, L. A., McLoughlin, L. & Crome, S. J. (2005).**Biodistribution and persistence of an MVA-vectored candidate HIV vaccine in SIV-infected rhesus macaques and SCID mice. *Vaccine* **23**, 1507-1514.

151. **Harari, A., Cellerai, C., Enders, F. B., Kostler, J., Codarri, L., Tapia, G., Boyman, O., Castro, E., Gaudieri, S., James, I., John, M., Wagner, R., Mallal, S. & Pantaleo, G. (2007).**Skewed association of polyfunctional antigen-specific CD8 T cell populations with HLA-B genotype. *Proc Natl Acad Sci U S A* **104**, 16233-16238.

152. **Harro, C., Sun, X., Stek, J. E., Leavitt, R. Y., Mehrotra, D. V., Wang, F., Bett, A. J., Casimiro, D. R., Shiver, J. W., Dinubile, M. J. & Quirk, E. (2009).**Safety and immunogenicity of the Merck adenovirus serotype 5 (MRKAd5) and MRKAd6 human immunodeficiency virus type 1 trigene vaccines alone and in combination in healthy adults. *Clin Vaccine Immunol* **16**, 1285-1292.

153. **Hart, T. K., Kirsh, R., Ellens, H., Sweet, R. W., Lambert, D. M., Petteway, S. R., Jr., Leary, J. & Bugelski, P. J. (1991).**Binding of soluble CD4 proteins to human immunodeficiency virus type 1 and infected cells induces release of envelope glycoprotein gp120. *Proc Natl Acad Sci U S A* **88**, 2189-2193.

154. **Harvey, T. J., Anraku, I., Linedale, R., Harrich, D., Mackenzie, J., Suhrbier, A. & Khromykh, A. A. (2003).**Kunjin virus replicon vectors for human immunodeficiency virus vaccine development. *J Virol* **77**, 7796-7803.

155. **Hawkridge, T., Scriba, T. J., Gelderbloem, S., Smit, E., Tameris, M., Moyo, S., Lang, T., Veldsman, A., Hatherill, M., Merwe, L., Fletcher, H. A., Mahomed, H., Hill, A. V., Hanekom, W. A., Hussey, G. D. & McShane, H. (2008).**Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in healthy adults in South Africa. *J Infect Dis* **198**, 544-552.

156. **Haynes, B. F., Fleming, J., St Clair, E. W., Katinger, H., Stiegler, G., Kunert, R., Robinson, J., Scearce, R. M., Plonk, K., Staats, H. F., Ortel, T. L., Liao, H. X. & Alam, S. M. (2005).**Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science* **308**, 1906-1908.

157. **Haynes, B. F., Telen, M. J., Hale, L. P. & Denning, S. M. (1989).**CD44--a molecule involved in leukocyte adherence and T-cell activation. *Immunol Today* **10**, 423-428.

158. **Hemelaar, J., Gouws, E., Ghys, P. D. & Osmanov, S. (2006).**Global and regional distribution of HIV-1

genetic subtypes and recombinants in 2004. *AIDS* **20**, W13-W23.

159. **Henderson, D. A. (1980).**Smallpox eradication. *Public Health Rep* **95**, 422-426.

160. **Henning, M. W. (1956).**Animal diseases in South Africa. Third edn: Central News Agency.

161. **Hessell, A. J., Hangartner, L., Hunter, M., Havenith, C. E., Beurskens, F. J., Bakker, J. M., Lanigan, C. M., Landucci, G., Forthal, D. N., Parren, P. W., Marx, P. A. & Burton, D. R. (2007).**Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* **449**, 101-104.

162. **Hessell, A. J., Poignard, P., Hunter, M., Hangartner, L., Tehrani, D. M., Bleeker, W. K., Parren, P. W., Marx, P. A. & Burton, D. R. (2009).**Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. *Nat Med* **15**, 951-954.

163. **Hill, A. V., Reece, W., Gothard, P., Moorthy, V., Roberts, M., Flanagan, K., Plebanski, M., Hannan, C., Hu, J. T., Anderson, R., Degano, P., Schneider, J., Prieur, E., Sheu, E. & Gilbert, S. C. (2000).**DNA-based vaccines for malaria: a heterologous prime-boost immunisation strategy. *Dev Biol (Basel)* **104**, 171-179.

164. **Hiller, G. & Weber, K. (1985).**Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *J Virol* **55**, 651-659.

165. **Hollinshead, M., Rodger, G., Van, E. H., Law, M., Hollinshead, R., Vaux, D. J. & Smith, G. L. (2001).**Vaccinia virus utilizes microtubules for movement to the cell surface. *J Cell Biol* **154**, 389-402.

166. **Hollinshead, M., Vanderplasschen, A., Smith, G. L. & Vaux, D. J. (1999).**Vaccinia virus intracellular mature virions contain only one lipid membrane. *J Virol* **73**, 1503-1517.

167. **Hsiao, J. C., Chung, C. S. & Chang, W. (1998).**Cell surface proteoglycans are necessary for A27L protein-mediated cell fusion: identification of the N-terminal region of A27L protein as the glycosaminoglycan-binding domain. *J Virol* **72**, 8374-8379.

168. **Hsiao, J. C., Chung, C. S. & Chang, W. (1999).**Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. *J Virol* **73**, 8750-8761.

169. **Huang, X., Lu, B., Yu, W., Fang, Q., Liu, L., Zhuang, K., Shen, T., Wang, H., Tian, P., Zhang, L. & Chen, Z. (2009).**A novel replication-competent vaccinia vector MVTT is superior to MVA for inducing high levels of neutralizing antibody via mucosal vaccination. *PLoS ONE* **4**, e4180.

170. **Huang, Y., Kong, W. P. & Nabel, G. J. (2001).**Human immunodeficiency virus type 1-specific immunity after genetic immunization is enhanced by modification of Gag and Pol expression. *J Virol* **75**, 4947-4951.

171. **Huber, M., Fischer, M., Misselwitz, B., Manrique, A., Kuster, H., Niederost, B., Weber, R., von, W., V, Gunthard, H. F. & Trkola, A. (2006).**Complement lysis activity in autologous plasma is associated with lower viral loads during the acute phase of HIV-1 infection. *PLoS Med* **3**, e441.

172. **Huber, M., von, W., V, Ammann, C. G., Kuster, H., Stiegler, G., Katinger, H., Weber, R., Fischer, M., Stoiber, H., Gunthard, H. F. & Trkola, A. (2008).**Potent human immunodeficiency virus-neutralizing and complement lysis activities of antibodies are not obligatorily linked. *J Virol* **82**, 3834-3842.

173. **Humbert, M., Rasmussen, R. A., Ong, H., Kaiser, F. M., Hu, S. L. & Ruprecht, R. M. (2008).**Inducing cross-clade neutralizing antibodies against HIV-1 by immunofocusing. *PLoS ONE* **3**, e3937.

174. **Hutchings, C. L., Birkett, A. J., Moore, A. C. & Hill, A. V. (2007).**Combination of protein and viral vaccines induces potent cellular and humoral immune responses and enhanced protection from murine malaria challenge. *Infect Immun* **75**, 5819-5826.

175. **Im, E. J. & Hanke, T. (2004).**MVA as a vector for vaccines against HIV-1. *Expert Rev Vaccines* **3**, S89-S97.

176. **Im, E. J., Nkolola, J. P., di, G. K., McMichael, A. J. & Hanke, T. (2006).**Induction of long-lasting multi-specific CD8+ T cells by a four-component DNA-MVA/HIVA-RENTA candidate HIV-1 vaccine in rhesus

macaques. *Eur J Immunol* **36**, 2574-2584.

177. **Jefferson, R. A., Burgess, S. M. & Hirsh, D. (1986).**beta-Glucuronidase from Escherichia coli as a gene-fusion marker. *Proc Natl Acad Sci U S A* **83**, 8447-8451.

178. **Jentarra, G. M., Heck, M. C., Youn, J. W., Kibler, K., Langland, J. O., Baskin, C. R., Ananieva, O., Chang, Y. & Jacobs, B. L. (2008).**Vaccinia viruses with mutations in the E3L gene as potential replication-competent, attenuated vaccines: scarification vaccination. *Vaccine* **26**, 2860-2872.

179. **Jia, B., Ng, S. K., DeGottardi, M. Q., Piatak, M., Yuste, E., Carville, A., Mansfield, K. G., Li, W., Richardson, B. A., Lifson, J. D. & Evans, D. T. (2009).**Immunization with single-cycle SIV significantly reduces viral loads after an intravenous challenge with SIV(mac)239. *PLoS Pathog* **5**, e1000272.

180. **Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J., Weinberger, L., Kostrikis, L. G., Zhang, L., Perelson, A. S. & Ho, D. D. (1999).**Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* **189**, 991-998.

181. **Johnson, P. R., Schnepp, B. C., Zhang, J., Connell, M. J., Greene, S. M., Yuste, E., Desrosiers, R. C. & Clark, K. R. (2009).**Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. *Nat Med* **15**, 901-906.

182. **Johnston, M. I. & Fauci, A. S. (2007).**An HIV vaccine--evolving concepts. *N Engl J Med* **356**, 2073-2081.

183. **Johnston, M. I. & Fauci, A. S. (2008).**An HIV vaccine--challenges and prospects. *N Engl J Med* **359**, 888-890.

184. **Jonckheere, H., Anne, J. & De, C. E. (2000).**The HIV-1 reverse transcription (RT) process as target for RT inhibitors. *Med Res Rev* **20**, 129-154.

185. **Jones, P. L., Korte, T. & Blumenthal, R. (1998).**Conformational changes in cell surface HIV-1 envelope glycoproteins are triggered by cooperation between cell surface CD4 and co-receptors. *J Biol Chem* **273**, 404-409.

186. **Kara, P. D., Afonso, C. L., Wallace, D. B., Kutish, G. F., Abolnik, C., Lu, Z., Vreede, F. T., Taljaard, L. C., Zsak, A., Viljoen, G. J. & Rock, D. L. (2003).**Comparative sequence analysis of the South African vaccine strain and two virulent field isolates of Lumpy skin disease virus. *Arch Virol* **148**, 1335-1356.

187. **Karnasuta, C., Paris, R. M., Cox, J. H., Nitayaphan, S., Pitisuttithum, P., Thongcharoen, P., Brown, A. E., Gurunathan, S., Tartaglia, J., Heyward, W. L., McNeil, J. G., Birx, D. L. & de Souza, M. S. (2005).**Antibody-dependent cell-mediated cytotoxic responses in participants enrolled in a phase I/II ALVAC-HIV/AIDSVAX B/E prime-boost HIV-1 vaccine trial in Thailand. *Vaccine* **23**, 2522-2529.

188. **Kates, J. R. & McAuslan, B. R. (1967).**Poxvirus DNA-dependent RNA polymerase. *Proc Natl Acad Sci U S A* **58**, 134-141.

189. **Keck, J. G., Baldick, C. J., Jr. & Moss, B. (1990).**Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. *Cell* **61**, 801-809.

190. **Kellogg, D. E. & Kwok, S. (1990).** Detection of human immunodeficiency virus. In *PCR Protocols: A Guide to Methods and Applications*, pp. 337-347. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White: Academic Press.

191. **Kent, S. J., Zhao, A., Best, S. J., Chandler, J. D., Boyle, D. B. & Ramshaw, I. A. (1998).**Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J Virol* **72**, 10180-10188.

192. **Kitabwalla, M., Ferrantelli, F., Wang, T., Chalmers, A., Katinger, H., Stiegler, G., Cavacini, L. A., Chou, T. C. & Ruprecht, R. M. (2003).**Primary African HIV clade A and D isolates: effective cross-clade neutralization with a quadruple combination of human monoclonal antibodies raised against clade B. *AIDS*

Res Hum Retroviruses **19**, 125-131.

193. **Kitching, R. P. (1986).**Passive protection of sheep against capripoxvirus. *Res Vet Sci* **41**, 247-250.

194. **Kitching, R. P., Bhat, P. P. & Black, D. N. (1989).**The characterization of African strains of capripoxvirus. *Epidemiol Infect* **102**, 335-343.

195. **Kitching, R. P., Hammond, J. M. & Taylor, W. P. (1987).**A single vaccine for the control of capripox infection in sheep and goats. *Res Vet Sci* **42**, 53-60.

196. **Kitching, R. P. & Mellor, P. S. (1986).**Insect transmission of capripoxvirus. *Res Vet Sci* **40**, 255-258.

197. **Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B. H., Wolinsky, S. & Bhattacharya, T. (2000).**Timing the ancestor of the HIV-1 pandemic strains. *Science* **288**, 1789-1796.

198. **Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C. & Ho, D. D. (1994).**Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* **68**, 4650-4655.

199. **Kovacs, G. R. & Moss, B. (1996).**The vaccinia virus H5R gene encodes late gene transcription factor 4: purification, cloning, and overexpression. *J Virol* **70**, 6796-6802.

200. **Kovacs, G. R., Rosales, R., Keck, J. G. & Moss, B. (1994).**Modification of the cascade model for regulation of vaccinia virus gene expression: purification of a prereplicative, late-stage-specific transcription factor. *J Virol* **68**, 3443-3447.

201. **Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W. C., Terwilliger, E., Dayton, A., Rosen, C., Haseltine, W. & Sodroski, J. (1987).**Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* **237**, 1351-1355.

202. **Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. & Hendrickson, W. A. (1998).**Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648-659.

203. **Lalvani, A. & Pareek, M. (2009).**Interferon gamma release assays: principles and practice. *Enferm Infecc Microbiol Clin*.

204. **Law, M., Carter, G. C., Roberts, K. L., Hollinshead, M. & Smith, G. L. (2006).**Ligand-induced and nonfusogenic dissolution of a viral membrane. *Proc Natl Acad Sci U S A* **103**, 5989-5994.

205. **Lemckert, A. A., Grimbergen, J., Smits, S., Hartkoorn, E., Holterman, L., Berkhout, B., Barouch, D. H., Vogels, R., Quax, P., Goudsmit, J. & Havenga, M. J. (2006).**Generation of a novel replication-incompetent adenoviral vector derived from human adenovirus type 49: manufacture on PER.C6 cells, tropism and immunogenicity. *J Gen Virol* **87**, 2891-2899.

206. **Lemckert, A. A., Sumida, S. M., Holterman, L., Vogels, R., Truitt, D. M., Lynch, D. M., Nanda, A., Ewald, B. A., Gorgone, D. A., Lifton, M. A., Goudsmit, J., Havenga, M. J. & Barouch, D. H. (2005).**Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol* **79**, 9694-9701.

207. **Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N. & Gregory, T. J. (1990).**Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* **265**, 10373-10382.

208. **Lesley, J., Hyman, R. & Kincade, P. W. (1993).**CD44 and its interaction with extracellular matrix. *Adv Immunol* **54**, 271-335.

209. **Lin, C. L., Chung, C. S., Heine, H. G. & Chang, W. (2000).**Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and *in vivo*. *J Virol* **74**, 3353-3365.

210. **Liu, J., Ewald, B. A., Lynch, D. M., Denholtz, M., Abbink, P., Lemckert, A. A., Carville, A.,**

Mansfield, K. G., Havenga, M. J., Goudsmit, J. & Barouch, D. H. (2008). Magnitude and phenotype of cellular immune responses elicited by recombinant adenovirus vectors and heterologous prime-boost regimens in rhesus monkeys. *J Virol* **82**, 4844-4852.

211. **Lu, S. (2009).**Heterologous prime-boost vaccination. *Curr Opin Immunol* **21**, 346-351.

212. **Lu, S., Santoro, J. C., Fuller, D. H., Haynes, J. R. & Robinson, H. L. (1995).**Use of DNAs expressing HIV-1 Env and noninfectious HIV-1 particles to raise antibody responses in mice. *Virology* **209**, 147-154.

213. **Lu, S., Wang, S. & Grimes-Serrano, J. M. (2008).**Current progress of DNA vaccine studies in humans. *Expert Rev Vaccines* **7**, 175-191.

214. **Luckay, A., Sidhu, M. K., Kjeken, R., Megati, S., Chong, S. Y., Roopchand, V., Garcia-Hand, D., Abdullah, R., Braun, R., Montefiori, D. C., Rosati, M., Felber, B. K., Pavlakis, G. N., Mathiesen, I., Israel, Z. R., Eldridge, J. H. & Egan, M. A. (2007).**Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. *J Virol* **81**, 5257-5269.

215. **Luthy, K. E., Tiedeman, M. E., Beckstrand, R. L. & Mills, D. A. (2006).**Safety of live-virus vaccines for children with immune deficiency. *J Am Acad Nurse Pract* **18**, 494-503.

216. **Luzzati, A. L., Giacomini, E., Giordani, L., Viora, M., Chersi, A., Camponeschi, B. & Pugliese, O. (1994).**An HIV p24 heptapeptide down-regulates antigen-specific responses in vitro interfering at the level of the T3-Ti complex. *Cell Immunol* **156**, 286-295.

217. **MacGregor, R. R., Boyer, J. D., Ciccarelli, R. B., Ginsberg, R. S. & Weiner, D. B. (2000).**Safety and immune responses to a DNA-based human immunodeficiency virus (HIV) type I env/rev vaccine in HIV-infected recipients: follow-up data. *J Infect Dis* **181**, 406.

218. **MacGregor, R. R., Boyer, J. D., Ugen, K. E., Lacy, K. E., Gluckman, S. J., Bagarazzi, M. L., Chattergoon, M. A., Baine, Y., Higgins, T. J., Ciccarelli, R. B., Coney, L. R., Ginsberg, R. S. & Weiner, D. B. (1998).**First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J Infect Dis* **178**, 92-100.

219. **Mach, B., Steimle, V., Martinez-Soria, E. & Reith, W. (1996).**Regulation of MHC class II genes: lessons from a disease. *Annu Rev Immunol* **14**, 301-331.

220. **Mack, T. M., Noble, J., Jr. & Thomas, D. B. (1972).**A prospective study of serum antibody and protection against smallpox. *Am J Trop Med Hyg* **21**, 214-218.

221. **Mackett, M., Smith, G. L. & Moss, B. (1982).**Vaccinia virus: a selectable eukaryotic cloning and expression vector. *Proc Natl Acad Sci U S A* **79**, 7415-7419.

222. **Mackett, M., Smith, G. L. & Moss, B. (1984).**General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. *J Virol* **49**, 857-864.

223. **Maecker, H. T. & Maino, V. C. (2003).**T cell immunity to HIV: defining parameters of protection. *Curr HIV Res* **1**, 249-259.

224. **Maggi, E., Macchia, D., Parronchi, P., Milo, D. & Romagnani, S. (1987).**Diminished production of interleukin 2 and gamma-interferon by cloned "T" cells from patients with the acquired immunodeficiency syndrome (AIDS). *Tumori* **73**, 273-278.

225. **Makedonas, G. & Betts, M. R. (2006).**Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection. *Springer Semin Immunopathol* **28**, 209-219.

226. **Malm, M., Rollman, E., Ustav, M., Hinkula, J., Krohn, K., Wahren, B. & Blazevic, V. (2005).**Cross-clade protection induced by human immunodeficiency virus-1 DNA immunogens expressing consensus sequences of multiple genes and epitopes from subtypes A, B, C, and FGH. *Viral Immunol* **18**, 678-688.

227. **Mansfield, K., Lang, S. M., Gauduin, M. C., Sanford, H. B., Lifson, J. D., Johnson, R. P. & Desrosiers, R. C. (2008).**Vaccine protection by live, attenuated simian immunodeficiency virus in the absence of high-titer antibody responses and high-frequency cellular immune responses measurable in the

periphery. *J Virol* **82**, 4135-4148.

228. **Mansky, L. M. & Temin, H. M. (1995).**Lower *in vivo* mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J Virol* **69**, 5087-5094.

229. **Mascola, J. R., Lewis, M. G., Stiegler, G., Harris, D., Vancott, T. C., Hayes, D., Louder, M. K., Brown, C. R., Sapan, C. V., Frankel, S. S., Lu, Y., Robb, M. L., Katinger, H. & Birx, D. L. (1999).**Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *J Virol* **73**, 4009-4018.

230. **Mascola, J. R., Sambor, A., Beaudry, K., Santra, S., Welcher, B., Louder, M. K., Vancott, T. C., Huang, Y., Chakrabarti, B. K., Kong, W. P., Yang, Z. Y., Xu, L., Montefiori, D. C., Nabel, G. J. & Letvin, N. L. (2005).**Neutralizing antibodies elicited by immunization of monkeys with DNA plasmids and recombinant adenoviral vectors expressing human immunodeficiency virus type 1 proteins. *J Virol* **79**, 771-779.

231. **Masemola, A., Mashishi, T., Khoury, G., Mohube, P., Mokgotho, P., Vardas, E., Colvin, M., Zijenah, L., Katzenstein, D., Musonda, R., Allen, S., Kumwenda, N., Taha, T., Gray, G., McIntyre, J., Karim, S. A., Sheppard, H. W. & Gray, C. M. (2004).**Hierarchical targeting of subtype C human immunodeficiency virus type 1 proteins by CD8+ T cells: correlation with viral load. *J Virol* **78**, 3233-3243.

232. **Masopust, D. (2009).**Developing an HIV cytotoxic T-lymphocyte vaccine: issues of CD8 T-cell quantity, quality and location. *J Intern Med* **265**, 125-137.

233. **McCutchan, F. E. (2000).**Understanding the genetic diversity of HIV-1. *AIDS* **14 Suppl 3**, S31-S44.

234. **McCutchan, F. E., Carr, J. K., Bajani, M., Sanders-Buell, E., Harry, T. O., Stoeckli, T. C., Robbins, K. E., Gashau, W., Nasidi, A., Janssens, W. & Kalish, M. L. (1999).**Subtype G and multiple forms of A/G intersubtype recombinant human immunodeficiency virus type 1 in Nigeria. *Virology* **254**, 226-234.

235. **McElrath, M. J., De Rosa, S. C., Moodie, Z., Dubey, S., Kierstead, L., Janes, H., Defawe, O. D., Carter, D. K., Hural, J., Akondy, R., Buchbinder, S. P., Robertson, M. N., Mehrotra, D. V., Self, S. G., Corey, L., Shiver, J. W. & Casimiro, D. R. (2008).**HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* **372**, 1894-1905.

236. **McKinnon, L. R., Ball, T. B., Kimani, J., Wachihi, C., Matu, L., Luo, M., Embree, J., Fowke, K. R. & Plummer, F. A. (2005).**Cross-clade CD8(+) T-cell responses with a preference for the predominant circulating clade. *J Acquir Immune Defic Syndr* **40**, 245-249.

237. **McShane, H. (2009).**Vaccine strategies against tuberculosis. *Swiss Med Wkly* **139**, 156-160.

238. **Mercer, A. A., Schmidt, A. & Weber, O. (2007).**Taxonomic structure of *Poxviridae* family. In *Poxviruses*, p. xvii-xx. Edited by A. A. Mercer, A. Schmidt & O. Weber: Birkha"user.

239. **Merchlinsky, M. (1990).**Mutational analysis of the resolution sequence of vaccinia virus DNA: essential sequence consists of two separate AT-rich regions highly conserved among poxviruses. *J Virol* **64**, 5029-5035.

240. **Merchlinsky, M. & Moss, B. (1989).**Nucleotide sequence required for resolution of the concatemer junction of vaccinia virus DNA. *J Virol* **63**, 4354-4361.

241. **Meyer, H., Sutter, G. & Mayr, A. (1991).**Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J Gen Virol* **72 (Pt 5)**, 1031-1038.

242. **Migueles, S. A., Sabbaghian, M. S., Shupert, W. L., Bettinotti, M. P., Marincola, F. M., Martino, L., Hallahan, C. W., Selig, S. M., Schwartz, D., Sullivan, J. & Connors, M. (2000).**HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* **97**, 2709-2714.

243. **Miller, M. D., Farnet, C. M. & Bushman, F. D. (1997).**Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J Virol* **71**, 5382-5390.

244. **Minke, J. M., Siger, L., Karaca, K., Austgen, L., Gordy, P., Bowen, R., Renshaw, R. W., Loosmore, S., Audonnet, J. C. & Nordgren, B. (2004).**Recombinant canarypoxvirus vaccine carrying the prM/E genes of West Nile virus protects horses against a West Nile virus-mosquito challenge. *Arch Virol Suppl*, 221-230.

245. **Miura, T., Brockman, M. A., Schneidewind, A., Lobritz, M., Pereyra, F., Rathod, A., Block, B. L., Brumme, Z. L., Brumme, C. J., Baker, B., Rothchild, A. C., Li, B., Trocha, A., Cutrell, E., Frahm, N., Brander, C., Toth, I., Arts, E. J., Allen, T. M. & Walker, B. D. (2009).**HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphotye recognition. *J Virol* **83**, 2743-2755.

246. **Moore, A. C. & Hill, A. V. (2004).**Progress in DNA-based heterologous prime-boost immunization strategies for malaria. *Immunol Rev* **199**, 126-143.

247. **Moore, A. C., Kong, W. P., Chakrabarti, B. K. & Nabel, G. J. (2002a).**Effects of antigen and genetic adjuvants on immune responses to human immunodeficiency virus DNA vaccines in mice. *J Virol* **76**, 243-250.

248. **Moore, C. B., John, M., James, I. R., Christiansen, F. T., Witt, C. S. & Mallal, S. A. (2002b).**Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* **296**, 1439-1443.

249. **Mosam, A. & Dlova, N. C. (2006).**HIV/AIDS in sub-Saharan Africa. *Dermatol Clin* **24**, 421-9, v.

250. **Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. (1986).**Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-2357.

251. **Moss, B. (1996).**Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc Natl Acad Sci U S A* **93**, 11341-11348.

252. **Moss, B. (2006).**Poxvirus entry and membrane fusion. *Virology* **344**, 48-54.

253. **Moss, B. & Rosenblum, E. N. (1973).**Letter: Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. *J Mol Biol* **81**, 267-269.

254. **Motomura, K., Kusagawa, S., Kato, K., Nohtomi, K., Lwin, H. H., Tun, K. M., Thwe, M., Oo, K. Y., Lwin, S., Kyaw, O., Zaw, M., Nagai, Y. & Takebe, Y. (2000).**Emergence of new forms of human immunodeficiency virus type 1 intersubtype recombinants in central Myanmar. *AIDS Res Hum Retroviruses* **16**, 1831-1843.

255. **Munyon, W., Paoletti, E. & Grace, J. T., Jr. (1967).**RNA polymerase activity in purified infectious vaccinia virus. *Proc Natl Acad Sci U S A* **58**, 2280-2287.

256. **Naito, T., Kaneko, Y. & Kozbor, D. (2007).**Oral vaccination with modified vaccinia virus Ankara attached covalently to TMPEG-modified cationic liposomes overcomes pre-existing poxvirus immunity from recombinant vaccinia immunization. *J Gen Virol* **88**, 61-70.

257. **Nayak, B. P., Sailaja, G. & Jabbar, A. M. (2006).**Augmenting the immunogenicity of DNA vaccines: role of plasmid-encoded Flt-3 ligand, as a molecular adjuvant in genetic vaccination. *Virology* **348**, 277-288.

258. **Ngichabe, C. K., Wamwayi, H. M., Barrett, T., Ndungu, E. K., Black, D. N. & Bostock, C. J. (1997).**Trial of a capripoxvirus-rinderpest recombinant vaccine in African cattle. *Epidemiol Infect* **118**, 63-70.

259. **Ngichabe, C. K., Wamwayi, H. M., Ndungu, E. K., Mirangi, P. K., Bostock, C. J., Black, D. N. & Barrett, T. (2002).**Long term immunity in African cattle vaccinated with a recombinant capripox-rinderpest virus vaccine. *Epidemiol Infect* **128**, 343-349.

260. **Nigam, P., Earl, P. L., Americo, J. L., Sharma, S., Wyatt, L. S., Edghill-Spano, Y., Chennareddi, L. S., Silvera, P., Moss, B., Robinson, H. L. & Amara, R. R. (2007).**DNA/MVA HIV-1/AIDS vaccine elicits long-lived vaccinia virus-specific immunity and confers protection against a lethal monkeypox challenge. *Virology* **366**, 73-83.

261. **Nishimura, Y., Igarashi, T., Haigwood, N. L., Sadjadpour, R., Donau, O. K., Buckler, C., Plishka, R. J., Buckler-White, A. & Martin, M. A. (2003).**Transfer of neutralizing IgG to macaques 6 h but not 24 h after SHIV infection confers sterilizing protection: implications for HIV-1 vaccine development. *Proc Natl Acad Sci U S A* **100**, 15131-15136.

262. **Nitayaphan, S., Pitisuttithum, P., Karnasuta, C., Eamsila, C., de, S. M., Morgan, P., Polonis, V., Benenson, M., Vancott, T., Ratto-Kim, S., Kim, J., Thapinta, D., Garner, R., Bussaratid, V., Singharaj, P., el-Habib, R., Gurunathan, S., Heyward, W., Birx, D., McNeil, J. & Brown, A. E. (2004).**Safety and immunogenicity of an HIV subtype B and E prime-boost vaccine combination in HIV-negative Thai adults. *J Infect Dis* **190**, 702-706.

263. **Nixon, D. F., Townsend, A. R., Elvin, J. G., Rizza, C. R., Gallwey, J. & McMichael, A. J. (1988).**HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature* **336**, 484-487.

264. **Okeke, M. I., Nilssen, O. & Traavik, T. (2006).**Modified vaccinia virus Ankara multiplies in rat IEC-6 cells and limited production of mature virions occurs in other mammalian cell lines. *J Gen Virol* **87**, 21-27.

265. **Panchanathan, V., Chaudhri, G. & Karupiah, G. (2006).**Protective immunity against secondary poxvirus infection is dependent on antibody but not on CD4 or CD8 T-cell function. *J Virol* **80**, 6333-6338.

266. **Panchanathan, V., Chaudhri, G. & Karupiah, G. (2008).**Correlates of protective immunity in poxvirus infection: where does antibody stand? *Immunol Cell Biol* **86**, 80-86.

267. **Panicali, D., Davis, S. W., Weinberg, R. L. & Paoletti, E. (1983).**Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin. *Proc Natl Acad Sci U S A* **80**, 5364-5368.

268. **Pantaleo, G., Menzo, S., Vaccarezza, M., Graziosi, C., Cohen, O. J., Demarest, J. F., Montefiori, D., Orenstein, J. M., Fox, C., Schrager, L. K. & . (1995).**Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N Engl J Med* **332**, 209-216.

269. **Pardo, M. C., Bauman, J. E. & Mackowiak, M. (1997).**Protection of dogs against canine distemper by vaccination with a canarypox virus recombinant expressing canine distemper virus fusion and hemagglutinin glycoproteins. *Am J Vet Res* **58**, 833-836.

270. **Parren, P. W., Marx, P. A., Hessell, A. J., Luckay, A., Harouse, J., Cheng-Mayer, C., Moore, J. P. & Burton, D. R. (2001).**Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. *J Virol* **75**, 8340-8347.

271. **Pastoret, P. P. & Brochier, B. (1996).**The development and use of a vaccinia-rabies recombinant oral vaccine for the control of wildlife rabies; a link between Jenner and Pasteur. *Epidemiol Infect* **116**, 235-240.

272. **Peeters, M. & Sharp, P. M. (2000).**Genetic diversity of HIV-1: the moving target. *AIDS* **14 Suppl 3**, S129-S140.

273. **Pereyra, F., Addo, M. M., Kaufmann, D. E., Liu, Y., Miura, T., Rathod, A., Baker, B., Trocha, A., Rosenberg, R., Mackey, E., Ueda, P., Lu, Z., Cohen, D., Wrin, T., Petropoulos, C. J., Rosenberg, E. S. & Walker, B. D. (2008).**Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Dis* **197**, 563-571.

274. **Perrin, A., Albina, E., Breard, E., Sailleau, C., Prome, S., Grillet, C., Kwiatek, O., Russo, P., Thiery, R., Zientara, S. & Cetre-Sossah, C. (2007).**Recombinant capripoxviruses expressing proteins of bluetongue virus: evaluation of immune responses and protection in small ruminants. *Vaccine* **25**, 6774-6783.

275. **Peters, P. J., Borst, J., Oorschot, V., Fukuda, M., Krahenbuhl, O., Tschopp, J., Slot, J. W. & Geuze, H. J. (1991).**Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J Exp Med* **173**, 1099-1109.

276. **Piguet, V., Schwartz, O., Le, G. S. & Trono, D. (1999).**The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. *Immunol Rev* **168**, 51-63.

277. **Pitisuttithum, P., Gilbert, P., Gurwith, M., Heyward, W., Martin, M., van, G. F., Hu, D., Tappero, J. W. & Choopanya, K. (2006).**Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis* **194**, 1661-1671.

278. **Piyasirisilp, S., McCutchan, F. E., Carr, J. K., Sanders-Buell, E., Liu, W., Chen, J., Wagner, R., Wolf, H., Shao, Y., Lai, S., Beyrer, C. & Yu, X. F. (2000).**A recent outbreak of human immunodeficiency virus type 1 infection in southern China was initiated by two highly homogeneous, geographically separated strains, circulating recombinant form AE and a novel BC recombinant. *J Virol* **74**, 11286-11295.

279. **Posner, M. R., Hideshima, T., Cannon, T., Mukherjee, M., Mayer, K. H. & Byrn, R. A. (1991).**An IgG human monoclonal antibody that reacts with HIV-1/GP120, inhibits virus binding to cells, and neutralizes infection. *J Immunol* **146**, 4325-4332.

280. **Poulet, H., Brunet, S., Boularand, C., Guiot, A. L., Leroy, V., Tartaglia, J., Minke, J., Audonnet, J. C. & Desmettre, P. (2003).**Efficacy of a canarypox virus-vectored vaccine against feline leukaemia. *Vet Rec* **153**, 141-145.

281. **Pretorius, A., van, K. M., Collins, N. E., Tshikudo, N., Louw, E., Faber, F. E., van Strijp, M. F. & Allsopp, B. A. (2008).**A heterologous prime/boost immunisation strategy protects against virulent E. ruminantium Welgevonden needle challenge but not against tick challenge. *Vaccine* **26**, 4363-4371.

282. **Prideaux, C. T., Kumar, S. & Boyle, D. B. (1990).**Comparative analysis of vaccinia virus promoter activity in fowlpox and vaccinia virus recombinants. *Virus Res* **16**, 43-57.

283. **Prieur, E., Gilbert, S. C., Schneider, J., Moore, A. C., Sheu, E. G., Goonetilleke, N., Robson, K. J. & Hill, A. V. (2004).**A Plasmodium falciparum candidate vaccine based on a six-antigen polyprotein encoded by recombinant poxviruses. *Proc Natl Acad Sci U S A* **101**, 290-295.

284. **Qiao, C., Jiang, Y., Tian, G., Wang, X., Li, C., Xin, X., Chen, H. & Yu, K. (2009).**Recombinant fowlpox virus vector-based vaccine completely protects chickens from H5N1 avian influenza virus. *Antiviral Res* **81**, 234-238.

285. **Qiao, C. L., Yu, K. Z., Jiang, Y. P., Jia, Y. Q., Tian, G. B., Liu, M., Deng, G. H., Wang, X. R., Meng, Q. W. & Tang, X. Y. (2003).**Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. *Avian Pathol* **32**, 25-32.

286. **Qiu, J. T., Chang, T. C., Lin, C. T., Chen, Y. M., Li, F. Q., Soong, Y. K. & Lai, C. H. (2007).**Novel codon-optimized GM-CSF gene as an adjuvant to enhance the immunity of a DNA vaccine against HIV-1 Gag. *Vaccine* **25**, 253-263.

287. **Quinn, T. C., Wawer, M. J., Sewankambo, N., Serwadda, D., Li, C., Wabwire-Mangen, F., Meehan, M. O., Lutalo, T. & Gray, R. H. (2000).**Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med* **342**, 921-929.

288. **Ranasinghe, C., Medveczky, J. C., Woltring, D., Gao, K., Thomson, S., Coupar, B. E., Boyle, D. B., Ramsay, A. J. & Ramshaw, I. A. (2006).**Evaluation of fowlpox-vaccinia virus prime-boost vaccine strategies for high-level mucosal and systemic immunity against HIV-1. *Vaccine* **24**, 5881-5895.

289. **Ranasinghe, C. & Ramshaw, I. A. (2009).**Genetic heterologous prime-boost vaccination strategies for improved systemic and mucosal immunity. *Expert Rev Vaccines* **8**, 1171-1181.

290. **Redfield, R. R., Wright, D. C., James, W. D., Jones, T. S., Brown, C. & Burke, D. S. (1987).**Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. *N Engl J Med* **316**, 673-676.

291. **Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Premsri, N., Namwat, C., de, S. M., Adams, E., Benenson, M., Gurunathan, S., Tartaglia, J., McNeil, J. G., Francis, D. P., Stablein, D., Birx, D. L., Chunsuttiwat, S., Khamboonruang, C., Thongcharoen, P., Robb, M. L., Michael, N. L., Kunasol, P. & Kim, J. H. (2009).**Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* **361**, 2209-2220.

292. **Rey, M. & Girard, M. P. (2008).**The global eradication of poliomyelitis: progress and problems. *Comp Immunol Microbiol Infect Dis* **31**, 317-325.

293. **Reynolds, M. R., Weiler, A. M., Weisgrau, K. L., Piaskowski, S. M., Furlott, J. R., Weinfurter, J. T., Kaizu, M., Soma, T., Leon, E. J., MacNair, C., Leaman, D. P., Zwick, M. B., Gostick, E., Musani, S. K., Price, D. A., Friedrich, T. C., Rakasz, E. G., Wilson, N. A., McDermott, A. B., Boyle, R., Allison, D. B., Burton, D. R., Koff, W. C. & Watkins, D. I. (2008).**Macaques vaccinated with live-attenuated SIV control replication of heterologous virus. *J Exp Med* **205**, 2537-2550.

294. **Rivas, P., Herrero, M. D., Puente, S., Ramirez-Olivencia, G. & Soriano, V. (2007).**Immunizations in HIV-infected adults. *AIDS Rev* **9**, 173-187.

295. **Robertson, D. L., Hahn, B. H. & Sharp, P. M. (1995).**Recombination in AIDS viruses. *J Mol Evol* **40**, 249-259.

296. **Robertson, M., Mehrotra, D., Fitzgerald, D., Duerr, A., Casimiro, D., McElrath, J., Lawrence, D. & Buchbinder, S. (2008).**Efficacy Results from the STEP Study (Merck V520 Protocol 023/HVTN 502): A Phase II Test-of-Concept Trial of the MRKAd5 HIV-1 Gag/Pol/Nef Trivalent Vaccine.

297. **Robinson, H. L., Montefiori, D. C., Johnson, R. P., Manson, K. H., Kalish, M. L., Lifson, J. D., Rizvi, T. A., Lu, S., Hu, S. L., Mazzara, G. P., Panicali, D. L., Herndon, J. G., Glickman, R., Candido, M. A., Lydy, S. L., Wyand, M. S. & McClure, H. M. (1999).**Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat Med* **5**, 526-534.

298. **Rodriguez, J. F. & Smith, G. L. (1990).**IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. *Nucleic Acids Res* **18**, 5347-5351.

299. **Romero, C. H., Barrett, T., Chamberlain, R. W., Kitching, R. P., Fleming, M. & Black, D. N. (1994a).**Recombinant capripoxvirus expressing the hemagglutinin protein gene of rinderpest virus: protection of cattle against rinderpest and lumpy skin disease viruses. *Virology* **204**, 425-429.

300. **Romero, C. H., Barrett, T., Evans, S. A., Kitching, R. P., Gershon, P. D., Bostock, C. & Black, D. N. (1993).**Single capripoxvirus recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. *Vaccine* **11**, 737-742.

301. **Romero, C. H., Barrett, T., Kitching, R. P., Carn, V. M. & Black, D. N. (1994b).**Protection of cattle against rinderpest and lumpy skin disease with a recombinant capripoxvirus expressing the fusion protein gene of rinderpest virus. *Vet Rec* **135**, 152-154.

302. **Rosel, J. L., Earl, P. L., Weir, J. P. & Moss, B. (1986).**Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the HindIII H genome fragment. *J Virol* **60**, 436-449.

303. **Rowland-Jones, S. L., Dong, T., Fowke, K. R., Kimani, J., Krausa, P., Newell, H., Blanchard, T., Ariyoshi, K., Oyugi, J., Ngugi, E., Bwayo, J., MacDonald, K. S., McMichael, A. J. & Plummer, F. A. (1998).**Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *J Clin Invest* **102**, 1758-1765.

304. **Ruprecht, R. M. (2009).**Passive Immunization with Human Neutralizing Monoclonal Antibodies Against HIV-1 in Macaque Models: Experimental Approaches. *Methods Mol Biol* **525**, 1-8.

305. **Russell, N. D., Graham, B. S., Keefer, M. C., McElrath, M. J., Self, S. G., Weinhold, K. J., Montefiori, D. C., Ferrari, G., Horton, H., Tomaras, G. D., Gurunathan, S., Baglyos, L., Frey, S. E., Mulligan, M. J., Harro, C. D., Buchbinder, S. P., Baden, L. R., Blattner, W. A., Koblin, B. A. & Corey, L. (2007).**Phase 2 study of an HIV-1 canarypox vaccine (vCP1452) alone and in combination with rgp120: negative results fail to trigger a phase 3 correlates trial. *J Acquir Immune Defic Syndr* **44**, 203-212.

306. **Sacha, J. B., Chung, C., Rakasz, E. G., Spencer, S. P., Jonas, A. K., Bean, A. T., Lee, W., Burwitz, B. J., Stephany, J. J., Loffredo, J. T., Allison, D. B., Adnan, S., Hoji, A., Wilson, N. A., Friedrich, T. C., Lifson, J. D., Yang, O. O. & Watkins, D. I. (2007).**Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression. *J Immunol* **178**, 2746-2754.

307. **Sadagopal, S., Amara, R. R., Montefiori, D. C., Wyatt, L. S., Staprans, S. I., Kozyr, N. L., McClure, H. M., Moss, B. & Robinson, H. L. (2005).**Signature for long-term vaccine-mediated control of a Simian and human immunodeficiency virus 89.6P challenge: stable low-breadth and low-frequency T-cell response capable of coproducing gamma interferon and interleukin-2. *J Virol* **79**, 3243-3253.

308. **Salzman, N. P. (1960).**The rate of formation of vaccinia deoxyribonucleic acid and vaccinia virus. *Virology* **10**, 150-152.

309. **Sambrook, L., Fritsch, E. F. & Maniatis, T. (1989).***Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbour Laboratory press.

310. **Sander, C. R., Pathan, A. A., Beveridge, N. E., Poulton, I., Minassian, A., Alder, N., Van, W. J., Hill, A. V., Gleeson, F. V., Davies, R. J., Pasvol, G. & McShane, H. (2009).**Safety and Immunogenicity of a New TB Vaccine, MVA85A, in M. tuberculosis Infected Individuals. *Am J Respir Crit Care Med*.

311. **Sanderson, C. M., Hollinshead, M. & Smith, G. L. (2000).**The vaccinia virus A27L protein is needed for the microtubule-dependent transport of intracellular mature virus particles. *J Gen Virol* **81**, 47-58.

312. **Sandstrom, E., Nilsson, C., Hejdeman, B., Brave, A., Bratt, G., Robb, M., Cox, J., Vancott, T., Marovich, M., Stout, R., Aboud, S., Bakari, M., Pallangyo, K., Ljungberg, K., Moss, B., Earl, P., Michael, N., Birx, D., Mhalu, F., Wahren, B. & Biberfeld, G. (2008).**Broad Immunogenicity of a Multigene, Multiclade HIV-1 DNA Vaccine Boosted with Heterologous HIV-1 Recombinant Modified Vaccinia Virus Ankara. *J Infect Dis*.

313. **Santra, S., Korber, B. T., Muldoon, M., Barouch, D. H., Nabel, G. J., Gao, F., Hahn, B. H., Haynes, B. F. & Letvin, N. L. (2008).**A centralized gene-based HIV-1 vaccine elicits broad cross-clade cellular immune responses in rhesus monkeys. *Proc Natl Acad Sci U S A* **105**, 10489-10494.

314. **Santra, S., Seaman, M. S., Xu, L., Barouch, D. H., Lord, C. I., Lifton, M. A., Gorgone, D. A., Beaudry, K. R., Svehla, K., Welcher, B., Chakrabarti, B. K., Huang, Y., Yang, Z. Y., Mascola, J. R., Nabel, G. J. & Letvin, N. L. (2005).**Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates. *J Virol* **79**, 6516-6522.

315. **Santra, S., Sun, Y., Parvani, J. G., Philippon, V., Wyand, M. S., Manson, K., Gomez-Yafal, A., Mazzara, G., Panicali, D., Markham, P. D., Montefiori, D. C. & Letvin, N. L. (2007).**Heterologous prime/boost immunization of rhesus monkeys by using diverse poxvirus vectors. *J Virol* **81**, 8563-8570.

316. **Saphire, E. O., Parren, P. W., Barbas, C. F., III, Burton, D. R. & Wilson, I. A. (2001).**Crystallization and preliminary structure determination of an intact human immunoglobulin, b12: an antibody that broadly neutralizes primary isolates of HIV-1. *Acta Crystallogr D Biol Crystallogr* **57**, 168-171.

317. **Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E. J., Shida, H., Hiller, G. & Griffiths, G. (1994).**Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. *J Virol* **68**, 130-147.

318. **Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L. & Reimann, K. A. (1999).**Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**, 857-860.

319. **Schnierle, B. S., Suezer, Y. & Sutter, G. (2007).**Recombinant poxvirus vaccines in biomedical research. In *Poxviruses*, pp. 311-327. Edited by A. A. Mercer, A. Schmidt & O. Weber: Birkha"user.

320. **Schramm, B. & Locker, J. K. (2005).**Cytoplasmic organization of POXvirus DNA replication. *Traffic* **6**, 839-846.

321. **Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. (2004).**Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**, 163-189.

322. **Seaman, M. S., Xu, L., Beaudry, K., Martin, K. L., Beddall, M. H., Miura, A., Sambor, A., Chakrabarti, B. K., Huang, Y., Bailer, R., Koup, R. A., Mascola, J. R., Nabel, G. J. & Letvin, N. L. (2005).**Multiclade human immunodeficiency virus type 1 envelope immunogens elicit broad cellular and humoral immunity in rhesus monkeys. *J Virol* **79**, 2956-2963.

323. **Senkevich, T. G. & Moss, B. (2005).**Vaccinia virus H2 protein is an essential component of a complex involved in virus entry and cell-cell fusion. *J Virol* **79**, 4744-4754.

324. **Senkevich, T. G., Ojeda, S., Townsley, A., Nelson, G. E. & Moss, B. (2005).**Poxvirus multiprotein entry-fusion complex. *Proc Natl Acad Sci U S A* **102**, 18572-18577.

325. **Shephard, E., Burgers, W. A., Van Harmelen, J. H., Monroe, J. E., Greenhalgh, T., Williamson, C. & Williamson, A. L. (2008).**A multigene HIV type 1 subtype C modified vaccinia Ankara (MVA) vaccine efficiently boosts immune responses to a DNA vaccine in mice. *AIDS Res Hum Retroviruses* **24**, 207-217.

326. **Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z. Q., Simon, A. J., Trigona, W. L., Dubey, S. A., Huang, L., Harris, V. A., Long, R. S., Liang, X., Handt, L., Schleif, W. A., Zhu, L., Freed, D. C., Persaud, N. V., Guan, L., Punt, K. S., Tang, A., Chen, M., Wilson, K. A., Collins, K. B., Heidecker, G. J., Fernandez, V. R., Perry, H. C., Joyce, J. G., Grimm, K. M., Cook, J. C., Keller, P. M., Kresock, D. S., Mach, H., Troutman, R. D., Isopi, L. A., Williams, D. M., Xu, Z., Bohannon, K. E., Volkin, D. B., Montefiori, D. C., Miura, A., Krivulka, G. R., Lifton, M. A., Kuroda, M. J., Schmitz, J. E., Letvin, N. L., Caulfield, M. J., Bett, A. J., Youil, R., Kaslow, D. C. & Emini, E. A. (2002).**Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**, 331-335.

327. **Shuman, S., Broyles, S. S. & Moss, B. (1987).**Purification and characterization of a transcription termination factor from vaccinia virions. *J Biol Chem* **262**, 12372-12380.

328. **Shuman, S. & Moss, B. (1987).**Identification of a vaccinia virus gene encoding a type I DNA topoisomerase. *Proc Natl Acad Sci U S A* **84**, 7478-7482.

329. **Siliciano, J. D., Kajdas, J., Finzi, D., Quinn, T. C., Chadwick, K., Margolick, J. B., Kovacs, C., Gange, S. J. & Siliciano, R. F. (2003).**Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat Med* **9**, 727-728.

330. **Smith, G. L. (2007).**Genus *Orthopoxvirus:Vaccinia virus*. In *Poxviruses*, pp. 1-45. Edited by A. A. Mercer, A. Schmidt & O. Weber: Birkha"user.

331. **Smith, G. L. & Moss, B. (1983).**Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene* **25**, 21-28.

332. **Smith, G. L., Vanderplasschen, A. & Law, M. (2002).**The formation and function of extracellular enveloped vaccinia virus. *J Gen Virol* **83**, 2915-2931.

333. **Smith, J. M., Amara, R. R., McClure, H. M., Patel, M., Sharma, S., Yi, H., Chennareddi, L., Herndon, J. G., Butera, S. T., Heneine, W., Ellenberger, D. L., Parekh, B., Earl, P. L., Wyatt, L. S., Moss, B. & Robinson, H. L. (2004).**Multiprotein HIV type 1 clade B DNA/MVA vaccine: construction, safety, and immunogenicity in Macaques. *AIDS Res Hum Retroviruses* **20**, 654-665.

334. **Someya, K., Cecilia, D., Ami, Y., Nakasone, T., Matsuo, K., Burda, S., Yamamoto, H., Yoshino, N., Kaizu, M., Ando, S., Okuda, K., Zolla-Pazner, S., Yamazaki, S., Yamamoto, N. & Honda, M. (2005).**Vaccination of rhesus macaques with recombinant Mycobacterium bovis bacillus Calmette-Guerin Env V3 elicits neutralizing antibody-mediated protection against simian-human immunodeficiency virus with a homologous but not a heterologous V3 motif. *J Virol* **79**, 1452-1462.

335. **Souza, A. P., Haut, L., Reyes-Sandoval, A. & Pinto, A. R. (2005).**Recombinant viruses as vaccines against viral diseases. *Braz J Med Biol Res* **38**, 509-522.

336. **Stasolla, C. & Thorpe, T. A. (2004).**Purine and pyrimidine nucleotide synthesis and degradation during in vitro morphogenesis of white spruce (Picea glauca). *Front Biosci* **9**, 1506-1519.

337. **Stickl, H., Hochstein-Mintzel, V., Mayr, A., Huber, H. C., Schafer, H. & Holzner, A. (1974).**[MVA vaccination against smallpox: clinical tests with an attenuated live vaccinia virus strain (MVA) (author's transl)]. *Dtsch Med Wochenschr* **99**, 2386-2392.

338. **Streeck, H., Lichterfeld, M., Alter, G., Meier, A., Teigen, N., Yassine-Diab, B., Sidhu, H. K., Little, S., Kelleher, A., Routy, J. P., Rosenberg, E. S., Sekaly, R. P., Walker, B. D. & Altfeld, M. (2007).**Recognition of a defined region within p24 gag by CD8+ T cells during primary human

immunodeficiency virus type 1 infection in individuals expressing protective HLA class I alleles. *J Virol* **81**, 7725-7731.

339. **Sun, J. C., Williams, M. A. & Bevan, M. J. (2004).**CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol* **5**, 927-933.

340. **Sutter, G. & Moss, B. (1992).**Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc Natl Acad Sci U S A* **89**, 10847-10851.

341. **Swigut, T., Shohdy, N. & Skowronski, J. (2001).**Mechanism for down-regulation of CD28 by Nef. *EMBO J* **20**, 1593-1604.

342. **Taylor, B. S., Sobieszczyk, M. E., McCutchan, F. E. & Hammer, S. M. (2008).**The challenge of HIV-1 subtype diversity. *N Engl J Med* **358**, 1590-1602.

343. **Taylor, G., Stott, E. J., Wertz, G. & Ball, A. (1991a).**Comparison of the virulence of wild-type thymidine kinase (tk)-deficient and tk+ phenotypes of vaccinia virus recombinants after intranasal inoculation of mice. *J Gen Virol* **72 (Pt 1)**, 125-130.

344. **Taylor, J., Meignier, B., Tartaglia, J., Languet, B., VanderHoeven, J., Franchini, G., Trimarchi, C. & Paoletti, E. (1995).**Biological and immunogenic properties of a canarypox-rabies recombinant, ALVAC-RG (vCP65) in non-avian species. *Vaccine* **13**, 539-549.

345. **Taylor, J., Trimarchi, C., Weinberg, R., Languet, B., Guillemin, F., Desmettre, P. & Paoletti, E. (1991b).**Efficacy studies on a canarypox-rabies recombinant virus. *Vaccine* **9**, 190-193.

346. **Thakar, M. R., Bhonge, L. S., Lakhashe, S. K., Shankarkumar, U., Sane, S. S., Kulkarni, S. S., Mahajan, B. A. & Paranjape, R. S. (2005).**Cytolytic T lymphocytes (CTLs) from HIV-1 subtype C-infected Indian patients recognize CTL epitopes from a conserved immunodominant region of HIV-1 Gag and Nef. *J Infect Dis* **192**, 749-759.

347. **Tollefsen, S., Tjelle, T., Schneider, J., Harboe, M., Wiker, H., Hewinson, G., Huygen, K. & Mathiesen, I. (2002).**Improved cellular and humoral immune responses against Mycobacterium tuberculosis antigens after intramuscular DNA immunisation combined with muscle electroporation. *Vaccine* **20**, 3370-3378.

348. **Tooze, J., Hollinshead, M., Reis, B., Radsak, K. & Kern, H. (1993).**Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *Eur J Cell Biol* **60**, 163-178.

349. **Tripathi, P. & Agrawal, S. (2007).**Immunobiology of human imunodeficiency virus infection. *Indian J Med Microbiol* **25**, 311-322.

350. **Trkola, A., Kuster, H., Rusert, P., von, W., V, Leemann, C., Weber, R., Stiegler, G., Katinger, H., Joos, B. & Gunthard, H. F. (2008).***In vivo* efficacy of human immunodeficiency virus neutralizing antibodies: estimates for protective titers. *J Virol* **82**, 1591-1599.

351. **Tulman, E. R., Afonso, C. L., Lu, Z., Zsak, L., Kutish, G. F. & Rock, D. L. (2001).**Genome of lumpy skin disease virus. *J Virol* **75**, 7122-7130.

352. **Tulman, E. R., Afonso, C. L., Lu, Z., Zsak, L., Kutish, G. F. & Rock, D. L. (2004).**The genome of canarypox virus. *J Virol* **78**, 353-366.

353. **UNAIDS (2009).**2009 AIDS Epidemic Update.

354. **Van Harmelen, J. H., Shephard, E., Thomas, R., Hanke, T., Williamson, A. L. & Williamson, C. (2003).**Construction and characterisation of a candidate HIV-1 subtype C DNA vaccine for South Africa. *Vaccine* **21**, 4380-4389.

355. **van Rooyen, P. J., Munz, E. K. & Weiss, K. E. (1969).**The optimal conditions for the multiplication of Neethling-type lumpy skin disease virus in embryonated eggs. *Onderstepoort J Vet Res* **36**, 165-174.

356. **Veazey, R. S., Shattock, R. J., Pope, M., Kirijan, J. C., Jones, J., Hu, Q., Ketas, T., Marx, P. A., Klasse, P. J., Burton, D. R. & Moore, J. P. (2003).**Prevention of virus transmission to macaque monkeys

by a vaginally applied monoclonal antibody to HIV-1 gp120. *Nat Med* **9**, 343-346.

357. **Vezys, V., Yates, A., Casey, K. A., Lanier, G., Ahmed, R., Antia, R. & Masopust, D. (2009).**Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* **457**, 196-199.

358. **Vijaysri, S., Jentarra, G., Heck, M. C., Mercer, A. A., McInnes, C. J. & Jacobs, B. L. (2008).**Vaccinia viruses with mutations in the E3L gene as potential replication-competent, attenuated vaccines: intra-nasal vaccination. *Vaccine* **26**, 664-676.

359. **Vuola, J. M., Keating, S., Webster, D. P., Berthoud, T., Dunachie, S., Gilbert, S. C. & Hill, A. V. (2005).**Differential immunogenicity of various heterologous prime-boost vaccine regimens using DNA and viral vectors in healthy volunteers. *J Immunol* **174**, 449-455.

360. **Walker, B. D. (2007a).**Elite control of HIV Infection: implications for vaccines and treatment. *Top HIV Med* **15**, 134-136.

361. **Walker, B. D. (2007b).**HIV controllers: an untapped source of clues to overcoming HIV infection. *Res Initiat Treat Action* **12**, 21-22.

362. **Walker, L. M., Phogat, S. K., Chan-Hui, P. Y., Wagner, D., Phung, P., Goss, J. L., Wrin, T., Simek, M. D., Fling, S., Mitcham, J. L., Lehrman, J. K., Priddy, F. H., Olsen, O. A., Frey, S. M., Hammond, P. W., Kaminsky, S., Zamb, T., Moyle, M., Koff, W. C., Poignard, P. & Burton, D. R. (2009).**Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* **326**, 285-289.

363. **Wallace, D. B. (1994).**Genomic characterization of southern African isolates of capripox and avipox viruses. Cape Town: University of Cape Town.

364. **Wallace, D. B., Ellis, C. E., Espach, A., Smith, S. J., Greyling, R. R. & Viljoen, G. J. (2006).**Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever. *Vaccine* **24**, 7181-7189.

365. **Wallace, D. B. & Viljoen, G. J. (2005).**Immune responses to recombinants of the South African vaccine strain of lumpy skin disease virus generated by using thymidine kinase gene insertion. *Vaccine* **23**, 3061-3067.

366. **Wallace, D. B., Weyer, J., Nel, L. H. & Viljoen, G. J. (2007).**Improved method for the generation and selection of homogeneous lumpy skin disease virus (SA-Neethling) recombinants. *J Virol Methods* **146**, 52-60.

367. **Wang, R., Doolan, D. L., Le, T. P., Hedstrom, R. C., Coonan, K. M., Charoenvit, Y., Jones, T. R., Hobart, P., Margalith, M., Ng, J., Weiss, W. R., Sedegah, M., de, T. C., Norman, J. A. & Hoffman, S. L. (1998).**Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* **282**, 476-480.

368. **Wang, Y. E., Li, B., Carlson, J. M., Streeck, H., Gladden, A. D., Goodman, R., Schneidewind, A., Power, K. A., Toth, I., Frahm, N., Alter, G., Brander, C., Carrington, M., Walker, B. D., Altfeld, M., Heckerman, D. & Allen, T. M. (2009).**Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1. *J Virol* **83**, 1845-1855.

369. **Wang, Z., La, R. C., Maas, R., Ly, H., Brewer, J., Mekhoubad, S., Daftarian, P., Longmate, J., Britt, W. J. & Diamond, D. J. (2004).**Recombinant modified vaccinia virus Ankara expressing a soluble form of glycoprotein B causes durable immunity and neutralizing antibodies against multiple strains of human cytomegalovirus. *J Virol* **78**, 3965-3976.

370. **Wei, C. M. & Moss, B. (1974).**Methylation of newly synthesized viral messenger RNA by an enzyme in vaccinia virus. *Proc Natl Acad Sci U S A* **71**, 3014-3018.

371. **Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., Hahn, B. H., Kwong, P. D. & Shaw, G. M. (2003).**Antibody neutralization and escape by HIV-1. *Nature* **422**, 307-312.

372. **Weiss, C. D., Levy, J. A. & White, J. M. (1990).**Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. *J Virol* **64**, 5674-5677.

373. **Weiss, K. E. (1968).**Lumpy skin disease virus. *Virology monographs*, 111-130.

374. **Weissenhorn, W., Dessen, A., Calder, L. J., Harrison, S. C., Skehel, J. J. & Wiley, D. C. (1999).**Structural basis for membrane fusion by enveloped viruses. *Mol Membr Biol* **16**, 3-9.

375. **Whitney, J. B. & Ruprecht, R. M. (2004).**Live attenuated HIV vaccines: pitfalls and prospects. *Curr Opin Infect Dis* **17**, 17-26.

376. **WHO (1980).**The Global Eradication of Smallpox. Final Report of the Global Commission for the Certification of Smallpox Eradication. History of International Public Health no. 4 edn. Geneva: World Health Organization.: WHO (World Health Organization).

377. **Widera, G., Austin, M., Rabussay, D., Goldbeck, C., Barnett, S. W., Chen, M., Leung, L., Otten, G. R., Thudium, K., Selby, M. J. & Ulmer, J. B. (2000).**Increased DNA vaccine delivery and immunogenicity by electroporation *in vivo*. *J Immunol* **164**, 4635-4640.

378. **Willey, S. & asa-Chapman, M. M. (2008).**Humoral immunity to HIV-1: neutralisation and antibody effector functions. *Trends Microbiol* **16**, 596-604.

379. **Williamson, C., Morris, L., Maughan, M. F., Ping, L. H., Dryga, S. A., Thomas, R., Reap, E. A., Cilliers, T., van, H. J., Pascual, A., Ramjee, G., Gray, G., Johnston, R., Karim, S. A. & Swanstrom, R. (2003).**Characterization and selection of HIV-1 subtype C isolates for use in vaccine development. *AIDS Res Hum Retroviruses* **19**, 133-144.

380. **Wilson, N. A., Reed, J., Napoe, G. S., Piaskowski, S., Szymanski, A., Furlott, J., Gonzalez, E. J., Yant, L. J., Maness, N. J., May, G. E., Soma, T., Reynolds, M. R., Rakasz, E., Rudersdorf, R., McDermott, A. B., O'Connor, D. H., Friedrich, T. C., Allison, D. B., Patki, A., Picker, L. J., Burton, D. R., Lin, J., Huang, L., Patel, D., Heindecker, G., Fan, J., Citron, M., Horton, M., Wang, F., Liang, X., Shiver, J. W., Casimiro, D. R. & Watkins, D. I. (2006).**Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. *J Virol* **80**, 5875-5885.

381. **Wittek, R., Menna, A., Muller, H. K., Schumperli, D., Boseley, P. G. & Wyler, R. (1978).**Inverted terminal repeats in rabbit poxvirus and vaccinia virus DNA. *J Virol* **28**, 171-181.

382. **Wright, C. F. & Coroneos, A. M. (1993).**Purification of the late transcription system of vaccinia virus: identification of a novel transcription factor. *J Virol* **67**, 7264-7270.

383. **Wright, C. F., Hubbs, A. E., Gunasinghe, S. K. & Oswald, B. W. (1998).**A vaccinia virus late transcription factor copurifies with a factor that binds to a viral late promoter and is complemented by extracts from uninfected HeLa cells. *J Virol* **72**, 1446-1451.

384. **Wu, L., Zhou, T., Yang, Z. Y., Svehla, K., O'Dell, S., Louder, M. K., Xu, L., Mascola, J. R., Burton, D. R., Hoxie, J. A., Doms, R. W., Kwong, P. D. & Nabel, G. J. (2009).**Enhanced exposure of the CD4-binding site to neutralizing antibodies by structural design of a membrane-anchored human immunodeficiency virus type 1 gp120 domain. *J Virol* **83**, 5077-5086.

385. **Wyand, M. S., Manson, K., Montefiori, D. C., Lifson, J. D., Johnson, R. P. & Desrosiers, R. C. (1999).**Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J Virol* **73**, 8356-8363.

386. **Wyand, M. S., Manson, K. H., Garcia-Moll, M., Montefiori, D. & Desrosiers, R. C. (1996).**Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J Virol* **70**, 3724-3733.

387. **Wyatt, L. S., Earl, P. L., Eller, L. A. & Moss, B. (2004).**Highly attenuated smallpox vaccine protects mice with and without immune deficiencies against pathogenic vaccinia virus challenge. *Proc Natl Acad Sci U S A* **101**, 4590-4595.

388. **Wyatt, L. S., Earl, P. L., Vogt, J., Eller, L. A., Chandran, D., Liu, J., Robinson, H. L. & Moss, B. (2008).**Correlation of immunogenicities and in vitro expression levels of recombinant modified vaccinia

virus Ankara HIV vaccines. *Vaccine* **26**, 486-493.

389. **Wyatt, R., Kwong, P. D., Desjardins, E., Sweet, R. W., Robinson, J., Hendrickson, W. A. & Sodroski, J. G. (1998).**The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393**, 705-711.

390. **Wyatt, R. & Sodroski, J. (1998).**The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**, 1884-1888.

391. **Xin, K. Q., Hamajima, K., Sasaki, S., Tsuji, T., Watabe, S., Okada, E. & Okuda, K. (1999).**IL-15 expression plasmid enhances cell-mediated immunity induced by an HIV-1 DNA vaccine. *Vaccine* **17**, 858-866.

392. **Xu, R., Megati, S., Roopchand, V., Luckay, A., Masood, A., Garcia-Hand, D., Rosati, M., Weiner, D. B., Felber, B. K., Pavlakis, G. N., Sidhu, M. K., Eldridge, J. H. & Egan, M. A. (2008).**Comparative ability of various plasmid-based cytokines and chemokines to adjuvant the activity of HIV plasmid DNA vaccines. *Vaccine* **26**, 4819-4829.

393. **Xu, W., Smith-Franklin, B. A., Li, P. L., Wood, C., He, J., Du, Q., Bhat, G. J., Kankasa, C., Katinger, H., Cavacini, L. A., Posner, M. R., Burton, D. R., Chou, T. C. & Ruprecht, R. M. (2001).**Potent neutralization of primary human immunodeficiency virus clade C isolates with a synergistic combination of human monoclonal antibodies raised against clade B. *J Hum Virol* **4**, 55-61.

394. **Yamamoto, H., Kawada, M., Takeda, A., Igarashi, H. & Matano, T. (2007).**Post-infection immunodeficiency virus control by neutralizing antibodies. *PLoS ONE* **2**, e540.

395. **Yang, O. O., Nguyen, P. T., Kalams, S. A., Dorfman, T., Gottlinger, H. G., Stewart, S., Chen, I. S., Threlkeld, S. & Walker, B. D. (2002).**Nef-mediated resistance of human immunodeficiency virus type 1 to antiviral cytotoxic T lymphocytes. *J Virol* **76**, 1626-1631.

396. **Yankee, T. M., Sheffer, D., Liu, Z., Dhillon, S., Jia, F., Chebloune, Y., Stephens, E. B. & Narayan, O. (2009).**Longitudinal study to assess the safety and efficacy of a live-attenuated SHIV vaccine in long term immunized rhesus macaques. *Virology* **383**, 103-111.

397. **Yeruham, I., Nir, O., Braverman, Y., Davidson, M., Grinstein, H., Haymovitch, M. & Zamir, O. (1995).**Spread of lumpy skin disease in Israeli dairy herds. *Vet Rec* **137**, 91-93.

398. **Yokoyama, T. & Mohri, S. (2008).**Prion diseases and emerging prion diseases. *Curr Med Chem* **15**, 912-916.

399. **Young, E., Basson, P. A. & Weiss, K. E. (1970).**Experimental infection of game animals with lumpy skin disease virus (prototype strain Neethling). *Onderstepoort J Vet Res* **37**, 79-87.

400. **Yu, S., Feng, X., Shu, T., Matano, T., Hasegawa, M., Wang, X., Ma, H., Li, H., Li, Z. & Zeng, Y. (2008).**Potent specific immune responses induced by prime-boost-boost strategies based on DNA, adenovirus, and Sendai virus vectors expressing gag gene of Chinese HIV-1 subtype B. *Vaccine* **26**, 6124-6131.

401. **Yuen, L., Davison, A. J. & Moss, B. (1987).**Early promoter-binding factor from vaccinia virions. *Proc Natl Acad Sci U S A* **84**, 6069-6073.

402. **Yuen, L. & Moss, B. (1987).**Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. *Proc Natl Acad Sci U S A* **84**, 6417-6421.

403. **Zhang, H., Huang, Y., Fayad, R., Spear, G. T. & Qiao, L. (2004).**Induction of mucosal and systemic neutralizing antibodies against human immunodeficiency virus type 1 (HIV-1) by oral immunization with bovine Papillomavirus-HIV-1 gp41 chimeric virus-like particles. *J Virol* **78**, 8342-8348.

404. **Zhang, W., Caspell, R., Karulin, A. Y., Ahmad, M., Haicheur, N., Abdelsalam, A., Johannesen, K., Vignard, V., Dudzik, P., Georgakopoulou, K., Mihaylova, A., Silina, K., Aptsiauri, N., Adams, V., Lehmann, P. V. & McArdle, S. (2009).**ELISPOT assays provide reproducible results among different laboratories for T-cell immune monitoring--even in hands of ELISPOT-inexperienced investigators. *J Immunotoxicol* **6**, 227-234.

405. **Zhang, X., Cassis-Ghavami, F., Eller, M., Currier, J., Slike, B. M., Chen, X., Tartaglia, J., Marovich, M. & Spearman, P. (2007).**Direct comparison of antigen production and induction of apoptosis by canarypox virus- and modified vaccinia virus ankara-human immunodeficiency virus vaccine vectors. *J Virol* **81**, 7022-7033.

406. **zur Megede J., Chen, M. C., Doe, B., Schaefer, M., Greer, C. E., Selby, M., Otten, G. R. & Barnett, S. W. (2000).**Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene. *J Virol* **74**, 2628-2635.

407. **Zwick, M. B., Jensen, R., Church, S., Wang, M., Stiegler, G., Kunert, R., Katinger, H. & Burton, D. R. (2005).**Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. *J Virol* **79**, 1252-1261.